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14. ABSTRACT
Purpose: The goal of the project is to determine the function of neuroendocrine (NE) cells in the initiation and progression of human prostate cancer
Scope: 1) Use a pten null mouse prostate cancer model to determine if ablation of NE cells by selective expression of a toxin in these cells can delay or prevent tumor initiation and/or progression. 2) Use a human tissue recombination model to determine if depletion of NE cells from human epithelial cells can retard the initiation and progression of the recombinant tumor. 3). Demonstrating the origin and molecular basis of human small cell carcinoma
Major findings: 1). We have successfully established a genotyping protocol to identify mice of the desired genotypes; 2). Expression of the toxin in tumor cells of TRAMP mice abolish tumor formation, demonstrating that the panned approach can ablate NE cells in mouse prostate tumors. 3). We have established a robust protocol to procure fresh human prostate tissue and isolate subpopulations of prostatic epithelial cells; 4). We have identified the appropriate combinations of cell surface markers for the isolation of NE cells; 5). Our initial study suggests that NE cells are not required for the propagation of established recombinant tumors.

15. SUBJECT TERMS
Prostate cancer, initiation, progression, neuroendocrine cells, animal model

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Introduction:

Despite years of study by clinicians and scientists around the world, there are still many unanswered questions in prostate cancer. A fundamental and clinically important issue is why prostate cancer responds to hormonal therapy initially but becomes resistant eventually in nearly all patients [1]. Prostate cancer is histologically heterogeneous consisting of luminal type tumor cells and a small component of neuroendocrine (NE) cells [2]. Unlike luminal type tumor cells that express androgen receptor (AR) and depend on androgen for proliferation, NE cells lack AR and are androgen-independent [3]. Hormonal therapy, while inhibiting luminal tumor cells, increases the number of NE cells in prostate cancer which is evident in recurrent castration-resistant prostate cancer[4]. In some patients, the recurrent tumor is composed of pure NE cells and is classified as small cell neuroendocrine carcinoma (SCNC) [5]. We hypothesize that NE cells play important roles in the initiation and progression of PC. We also hypothesize that they are the cells of origin for SCNC and p53 is the molecular target. This research proposal has the following specific aims: 1: To determine if NE cells are required for tumor initiation and/or progression in a mouse PC model; 2: To determine if NE cells are required for tumor initiation and/or progression in a human PC model; 3: Cell of origin and the molecular targets of prostatic small cell neuroendocrine carcinoma

Body:

Research accomplishments associated with Task 1: In this task, we will generate *pten*$^{loxP/loxP}$/pb-Cre/CR2-toxin+ mice by breeding pten conditional knockout mice with CR2-toxin mice. We will then observe tumor development and whether the mice develop castration-resistant tumors after castration (Time frame: Months 1 – 36)

1a: Breeding and genotyping (Time frame: Months 1 – 24)

Our goal is to determine the function of neuroendocrine cells in the initiation and progression of prostate cancer. Our approach is to cross pten-/- mice with CR2-toxin mice to generate *pten*$^{loxP/loxP}$/pb-Cre/CR2-toxin+ mice. The hypothesis is that in the male mice, the toxin will be expressed in prostate neuroendocrine cells because of the selective activity of CR2 promoter in such cells [6-7], resulting in ablation of the neuroendocrine cells. This will give us an opportunity to definitively determine the function of neuroendocrine cells in prostate cancer.

We have successfully established genotyping protocol for identifying mice that are of the desired genotype. As can be seen from Figure 1, mice with prostate deletion of *Pten* can be identified through PCR by 3 primers: WT forward 5'TCCCAGAGTTCATACCAGGA3', WT reverse 5'GCAATGGCCAGTACTAGTGAAC3' and an internal primer 5'AATCTGTGCATGAAGGGAAC3'. For *Probasin-Cre* detection, we used the following primers: 5'CAAAACAGGTAGTTATTCGG3' and 5'CGTATAGCCGAAATTGCCAG3'. For detection of *CR2-toxin*, we used two rounds

of PCR. In the first round, the following primers were used: 5'cttaacgctttcgcctgttc3' and 5'tcgtaccacgggactaaacc3'. The product of the first PCR reaction was diluted 20 fold and used as the input for the 2nd round of PCR with the primers: 5'gctctctggaaaagctggag3', 5'agggaaggctgagcactaca3'.

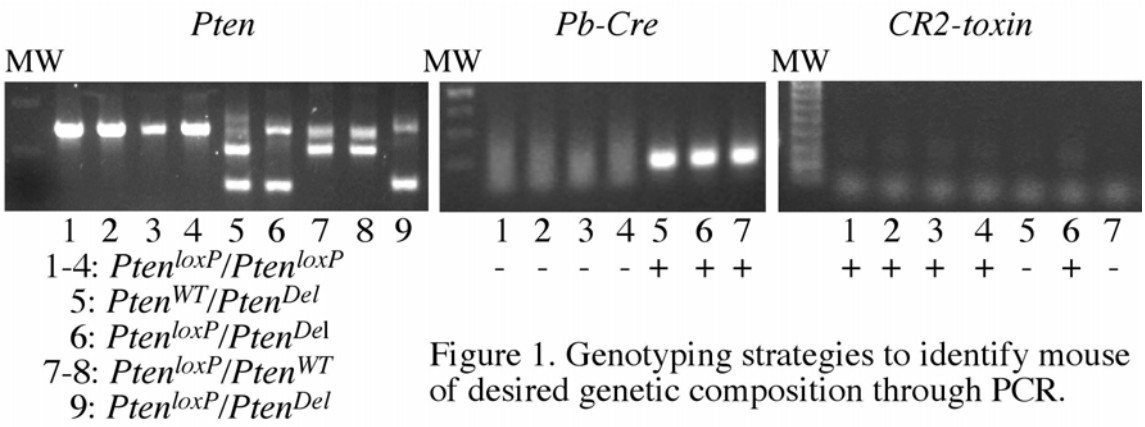

Figure 1. Genotyping strategies to identify mouse of desired genetic composition through PCR.

1b: Observing the development of primary tumors (Time frame: Months 12 – 24)

Although pten-/- model will be used to demonstrate the function of neuroendocrine cells, an important control is the TRAMP (transgenic adenocarcinoma of the mouse prostate) mouse prostate cancer model. Prostate tumors in TRAMP mice are composed exclusively of neuroendocrine cells. Therefore this model is a perfect control for us to demonstrate if expression of toxin can abolish the neuroendocrine cells and delay or prevent the development of the tumor as a result.

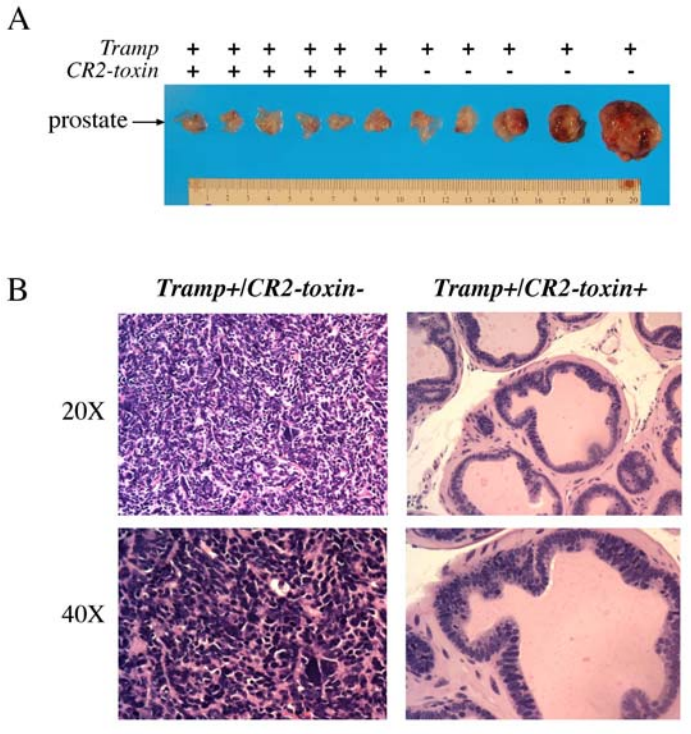

Figure 2. CR2-toxin expression blocks the small cell carcinoma development in mice induced by TRAMP expression. A. Prostates from mice with indicated genotypes. B. H&E staining of the prostate pathology with Tramp and CR2-toxin expression.

Therefore, we have crossed TRAMP mice with CR2-toxin mice and generated TRAMP mice with or without the expression of CR2-toxin. As shown in Figure 2A, mice without CR2-toxin expression develops aggressive tumor whose pathology is consistent with small cell neuroendocrine carcinoma (Figure 2B). However, in mice with co-expression of CR2-toxin, there is no visible tumor formation upon dissection, which is supported by histological examination (Figure 2B). These results demonstrate that our approach to abolish

neuroendocrine cells in the prostate with CR2 promoter-driven diphtheria toxin is a valid strategy, which will help us to definitively determine if neuroendocrine cells play a role in prostate cancer development and progression.

At the moment, we do not have sufficient number of pten-/- mice with the desired genotype to conclude whether prostatic neuroendocrine cells contribute to the development of prostatic adenocarcinoma as well as castration-resistant prostate cancer in this animal model. We are continuing collecting mice with the desired genotype and hope to complete this part soon.

1c: Castration and observation of the development of castration-resistant tumors (Time frame: Months 24-36)

To be performed

Research accomplishments associated with Task 2: In this task, we will procure fresh human prostate cancer tissue, separate tumor from benign prostate, separate epithelial cells into NE and non-NE cells, and perform tissue regeneration experiments to determine if NE cells are essential in tumor initiation and progression

2a. Procurement of fresh human prostate cancer tissue, separate tumor from benign prostate, separate tumor cells into NE and non-NE tumor cells (Time frame: Months 1 – 36)

We have established a robust system for the procurement of fresh prostate tissue, involving close collaborations among urologists, pathologists, Pathologist Assistants, technical staff from UCLA's tissue pathology core laboratory (TPCL) and basic researchers [8]. There is a seamless workflow starting from when the prostate is removed from the patients and including rapid transportation of the specimen to pathology, gross examination of the prostate, procurement of tissue for research, diagnosis of the procured tissue, separation of the tissue into benign prostate and prostate cancer, preparation of single cell suspension and flow cytometric separation of sub-population of epithelial cells based on cell surface markers. We have published as a high profile article describing the technology developed by our group (Goldstein et al. Nat Protoc. 2011; 6:656-67).

We explored the utility of several candidate cell-surface markers for the isolation/purification of an enriched subset of neuroendocrine cells using Fluorescence Activated Cell Sorting (FACS) on dissociated human prostate tissue preparations. After significant attempts and optimization, we identified the antigen CD56/NCAM (Neural Cell Adhesion Molecule) as the most robust marker for NE cells in primary human prostate. Our gating strategy was based on a negative depletion for the hematopoietic cell-surface marker CD45, positive enrichment for the epithelial antigens EpCAM (Trop1) or Trop2, and further separation into CD56+ (NE-enriched) and CD56- (NE-depleted) cells. Quantitative PCR analysis for classical NE genes including chromogranin

A and Neuron-Specific Enolase (NSE) demonstrated significant enrichment for NE-specific transcripts in the CD56+ fraction compared to the CD56- subset.

2b. Tissue regeneration experiment to determine if NE cells are involved in tumor initiation (Time frame: Months 1 – 24)

To be performed

2c. Serial transplantation and tissue regeneration to determine if NE cells are involved in tumor progression

Regardless of the role of NE cells in the initiation of prostate cancer, NE cells are continually found in tumors suggesting a role in tumor progression and maintenance [9]. We have recently established a new model of aggressive primary human prostate cancer. Naïve benign epithelial cells are transduced with the oncogenes Myc and AKT, combined with UGSM cells and transplanted in vivo into immune-deficient mice. We utilize an activated form of AKT (myristoylated rendering it membrane-bound) which promotes activation of the PI3-Kinase pathway, commonly found in human prostate cancer through loss of the tumor-suppressor PTEN or related mechanisms. Myc is rarely mutated or amplified in human prostate cancer, but elevated levels of nuclear Myc protein are extremely common especially at the early stages of prostate cancer initiation. Therefore

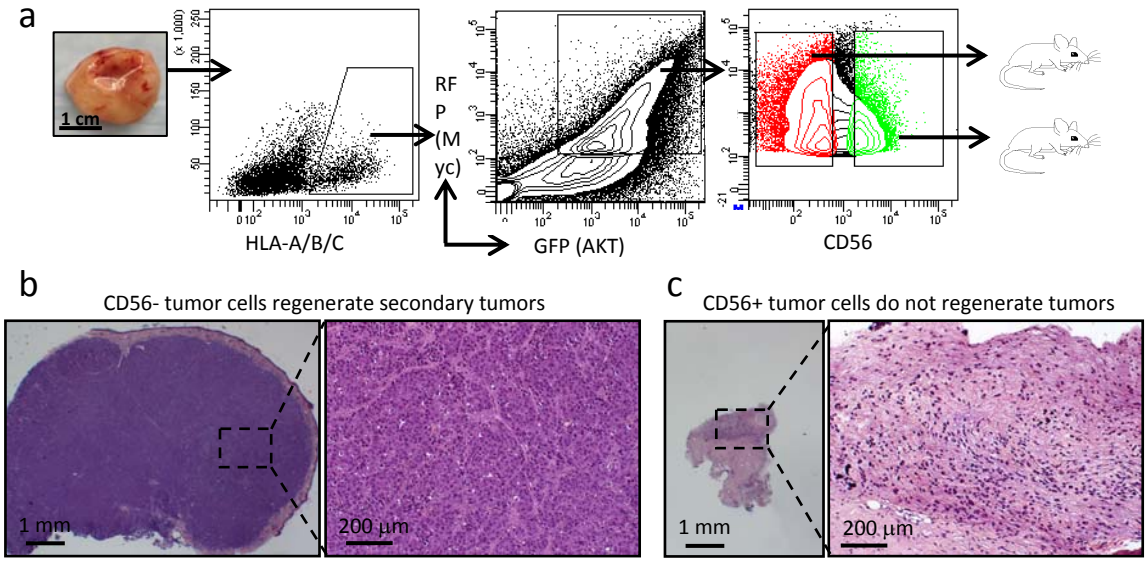

Fig. 3. a. Tumors initiated from naïve human prostate basal cells expressing Myc and AKT are dissociated to single cells, stained with a pan-HLA-A/B/C human antibody and gated based on HLA+, GFP+/RFP+ from lentivirus carrying oncogenes AKT (GFP) and Myc (RFP), and further sorted into CD56+ and CD56- subsets. Isolated subsets are transplanted back into recipient mice and harvested 6-12 weeks later. **b**, A representative secondary tumor derived from CD56- tumor cells is shown, stained for hematoxylin and eosin. Tumors formed consistently from the transplantation of 5000 CD56- cells (5 tumors per 5 transplants). **c**, A representative graft comprised of mesenchymal cells without a detectable tumor is shown as only one tumor formed out of five transplantations of 5000 CD56+ cells.

we engineered increased levels of wild-type Myc in addition to activated AKT in naïve benign cells. While high levels of Myc alone or AKT alone were not sufficient to drive full progression to cancer, the combination synergized to initiate large highly-proliferative tumors. Dissociated tumor cells were capable of propagating adenocarcinoma upon transplantation into mice. Tumors maintained a phenotype that was Keratin 8+ and p63- indicating an acinar-type or luminal-like cell. Importantly, staining for chromogranin A indicated continued presence of NE cells in tumors. Therefore, we separated out the CD56+ and CD56- fraction from aggressive prostate tumors initiated by Myc and AKT, and transplanted each subset into mice. After 12 weeks, only the CD56- (NE-depleted) fraction could initiate tumors, demonstrating that in this model, NE cells may not be required for tumor propagation (Fig. 3)

Research accomplishments associated with Task 3: In this task, we will procure fresh human prostate cancer tissue, separate tumor from benign prostate, separate epithelial cells into NE and non-NE cells, and perform tissue regeneration experiment to determine if SV40 T antigen induces SCNC in NE cells and if p53 is the molecular targets

3a: Same as 2a

3b. Tissue regeneration experiment to determine if NE cells are the cells of origin for SCNC (Time frame: Months 1 – 24)

Dissociated naïve benign human prostate tissue was separated by FACS into CD56+ (NE-enriched) and CD56- (NE-depleted) fractions and transduced with lentivirus carrying the SV40 Large T-antigen. Transduced cells were combined with UGSM and transplanted into mice in vivo. Dissociated cells from two different patients were tested and no growths were established from either the CD56+ or CD56- fraction. These findings suggest two possible outcomes. First, the quality of the tissue from these patients may not have been sufficient for continued growth of the cells in the new hosts. Working with primary human tissue can be challenging as the length of time that tissues are kept without a blood supply before being utilized for research can vary greatly. The second possibility is that the SV40 T-antigen is toxic to naïve benign primary human prostate cells when introduced through lentiviral transduction.

3c. Tissue regeneration experiment to determine if p53 is the molecular target for SCNC (Time frame: Months 12 – 36)

To be performed

<u>Key Research Accomplishments</u>

1). We have successfully established a genotyping protocol to identify mice of the desired genotypes.

2). Expression of the toxin in tumor cells of TRAMP mice abolishes tumor formation, demonstrating that the panned approach can ablate NE cells in mouse prostate tumors.

3). We have established a robust protocol to procure fresh human prostate tissue and isolate subpopulations of prostatic epithelial cells.
4). We have identified the appropriate combinations of cell surface markers for the isolation of NE cells.
5). Our initial study suggests that NE cells are not required for the propagation of established recombinant tumors.

Reportable outcomes

Manuscripts, abstracts, presentations;

Manuscripts:

1. Goldstein AS, Drake JM, Burnes DL, Finley DS, Zhang H, Reiter RE, Huang J, and Witte ON. Methods for the purification and direct transformation of epithelial progenitor cells from primary human prostate. Nature Protocol. 2011; 6:656-67. PMID: 21527922
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3. Cheng L, Zhang S, Alexander R, Yao Y, MacLennan GT, Pan CX, **Huang J**, Wang M, Montironi R, Lopez-Beltran A: The landscape of *EGFR* pathways and personalized management of nonsmall cell lung cancer. **Future Oncology** 2011; 7: 519-41. PMID: 21463141
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11. Goldstein AS and **Huang J**. Do neuroendocrine cells come up large in small cell lung cancer? **Cell Cycle** 2011; 2011 Nov 1;10(21). PMID: 22024918
12. Tan N, Margolis DJA, McClure TD, Finley DS, Reiter RE, **Huang J**, Raman SS. Radical prostatectomy: value of prostate MRI in surgical planning. **Abdom Imaging**, 2011 Oct 13. [Epub ahead of print]. PMID: 2199356
13. Finley DS, Margolis D, Raman SS, Ellingson BM, Natarajan S, Tan N, **Huang J**, Reiter RE. Fine-tuning robot-assisted radical prostatectomy planning with MRI. **Urol Oncol**. 2011 Sep 7. [Epub ahead of print] PMID: 21906964
14. Huang L, Schauer IG, Zhang J, Mercado-Uribe I, Deavers MT, **Huang J**, Liu J. The oncogenic gene fusion TMPRSS2: ERG is not a diagnostic or prognostic marker for ovarian cancer. **Int J Clin Exp Pathol.** 2011;4:644-50. PMID: 22076164
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Presentations:

Invited by academic institutions:

1. Neuroendocrine Differentiation in Prostate Cancer, UCLA Specialized Program of Research Excellence (SPORE) in Prostate Cancer, April 2011
2. Pathology of Prostate Cancer, Wu Jie-Ping Urologic Center, Beijing University, May 2011
3. Neuroendocrine Differentiation in Prostate Cancer, Anhui Medical University, May 2011
4. Immunohistochemistry in the Differential Diagnosis of Genitourinary Tumors, Anhui Medical University, May 2011
5. Neuroendocrine Differentiation in Prostate Cancer, Department of Urology, Massachusetts General Hospital of Harvard University, June 2011
6. Immunohistochemistry in the Diagnosis and Differential Diagnosis of Genitourinary Tumors. Adicon Clinical Laboratories Inc., Hangzhou, China, November 1, 2011
7. Medical Education in the United States, Anhui Medical University, Hefei, China. November 8, 2011
8. Medical-Legal Issues in the United States, the First Affiliated Hospital of Anhui medical University, Hefei, China, November 10, 2011

Invited by local, national or international conferences:

1. Individualized treatment for cancer. Invited Speaker. Technical Symposium of 49th anniversary of the Chinese-American Engineers and Scientists Association of Southern California (CESASC). San Gabriel, CA, April 2011
2. Tissue recombination technology in the study of tumor initiation cells for prostate cancer. Invited Speaker. Steering Committee Meeting, Intestinal Stem Cell Consortium (ISCC). Stowers Institute, Kansas City, Missouri, May 2011
3. Diagnostic Usage of Immunohistochemistry in Genitourinary Pathology. 4th Biannual MD Anderson Cancer Center-Fudan University Pathology Conference. May 14-15, 2011. Shanghai, China.
4. International session moderator, 18th Annual Meeting of Chinese Urological Association, October 28-30, 2011. Nanjing, China
5. Speaker for Cancer Committee, Zhejiang University-UCLA Joint Center for Research, Hangzhou, China. November 4, 2011
6. Recent Progress in Prostate cancer, Continuing Medical Education Conference of Chinese Urological Association, Huangshan, China, November 12, 2011
7. Panel Member, NCI-MMHCC Pathology Consensus Report Workshop, April 17-18, 2012, New York, NY
8. Pathology of prostate cancer. 2012 Hangzhou International Pathology Symposium, Hangzhou, China, April, 2012
9. Pathology of testicular tumors. 2012 Hangzhou International Pathology Symposium, Hangzhou, China, April, 2012
10. Small cell carcinoma of the prostate: Molecular mechanisms of carcinogenesis. The 7th Forum of Prostate Disease. Shanghai, China, June 2012.

Licenses applied for and/or issued:

Patent filed:

USSN 61/597,098: Combinatorial drug treatment for castration-resistant prostate cancer

Funding applied for based on work supported by this award

1. Cal-Tech-UCLA Joint Center for Translational Medicine Program (PI Huang)
   Period: 6/1/2011 – 5/31/2012
   Title: Biomarkers for prostate cancer
   Role: Principal Investigator

2. Translational Research Funds, UCLA Department of Pathology (PI: Kerkoutian)
   Period: 10/01/11 – 09/31/12
   Title: Predicting the Aggressiveness of Prostate Cancer on Biopsy

CONCLUSION:

We have demonstrated that expression of toxin is an appropriate approach to ablate neuroendocrine cells in mouse models of prostate cancer, which will demonstrate if such cells play important roles in the initiation and progression of prostate cancer. We have established a robust system to procure fresh human prostate tissue and isolate subpopulations of epithelial cells including neuroendocrine cells. Our initial study

suggests that neuroendocrine cells may not be required for the propagation of established tumors in xenograft models. Further studies in genetic and recombinant mouse models of prostate cancer will demonstrate the function of neuroendocrine cells in human prostate cancer.

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1. Scher, H.I. and C.L. Sawyers, *Biology of progressive, castration-resistant prostate cancer: directed therapies targeting the androgen-receptor signaling axis.* J Clin Oncol, 2005. **23**(32): p. 8253-61.
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9. Huang, J. and P.A. diSant'Agnese, *Neuroendocrine differentiation in prostate cancer: An overview.*, in *Advances in Oncology: The Expanding Role of Octreotide.* , S. Lamberts, Editor. 2002, BioScientifica Ltd: Bristol. p. 243-262.

Appendices:

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Purification and direct transformation of epithelial progenitor cells from primary human prostate

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Abstract

Epithelial cell transformation has been demonstrated in numerous animal models for the study of solid tumor biology. However, little evidence exists for human epithelial cell transformation without prior immortalization via genetic influences such as SV40 T-antigen, limiting our knowledge of the events that can transform naïve human epithelium. Here we describe a system developed in our lab to directly transform freshly-isolated primary human prostate epithelial cells without prior culture or immortalization. Prostate tissue is obtained from patients, and benign tissue is separated from cancer. Benign and cancer tissue are mechanically and enzymatically dissociated to single cells overnight, and immune cells and epithelial subsets are isolated based on differential expression of surface antigens. Epithelial progenitor cells are transduced with lentiviruses expressing oncogenes and combined with inductive stroma for in vivo studies. 8-16 weeks after transplantion into immune-deficient mice, the development of lesions histologically classified as benign prostate, prostatic intraepithelial neoplasia (PIN) and adenocarcinoma can be evaluated.

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*These authors contributed equally to the manuscript.

AUTHOR CONTRIBUTIONS A.S.G., D.L.B., H.Z., J.H., R.E.R. and O.N.W. contributed to protocol development. A.S.G, J.M.D., D.L.B., and H.Z. generated the figures. A.S.G., J.M.D., D.S.F., and O.N.W. wrote the paper.

COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

Keywords

Prostate cancer; Human regeneration; Human prostate; Flow cytometry; Lentivirus

INTRODUCTION

Understanding the genetic and cellular origins of human prostate cancer is a primary focus of interventional inquiry. Human prostate cancer is commonly regarded as a disease of luminal cell origin based on pathological evidence and immunohistochemical markers. Recent findings, however, complicate the picture and suggest that basal stem-like cells are a potential cell of origin[1-3]. The unique ability to self-renew suggests that tissue stem cells may serve as a likely origin of multiple cancers, making these cells intriguing targets for investigative research and therapeutic intervention[4, 5]. Therefore, delineating the epithelial hierarchy and purifying specific cell types from prostate and other tissues based on functional characteristics will help researchers to examine the cellular contexts and genetic influences undermining epithelial transformation.

Overview of the procedure

This protocol describes how to prepare, purify, and transform defined populations of primary human prostate epithelial cells and has been used recently to identify a cell of origin for human prostate cancer[2]. A sequential enzymatic digestion of primary tissue yields a total dissociated cell preparation containing all of the cell-types present in the original tissue, with the sole exception of red blood cells removed by a specific lysis step. We use Fluorescence Activated Cell Sorting (FACS) techniques to purify immune and epithelial cell subsets. This approach allows for the simultaneous isolation of distinct cell populations. Other methodologies to purify epithelial progenitor cells rely on differential centrifugation and/or preferential adhesion of specific cell types to extracellular matrices such as collagen[6, 7]. These protocols may be useful to enrich for epithelium or purify distinct subpopulations, but these methods leave other cells behind, whether they are stroma or stem cell-depleted epithelial populations. In contrast to magnetic bead separation, FACS provides precise control to purify cells that express high, intermediate, or low levels of multiple antigens. We have utilized this strategy for the purification of human prostate epithelial progenitor cells that form spheres[8], tubules, and malignant structures resembling prostate cancer[2] while also allowing for simultaneous isolation and discrimination of the remaining epithelial and non-epithelial subsets, including immune cells.

Purified epithelial cells are transduced with lentivirus to over-express different oncogenes, and transplanted into immune-deficient mice for regeneration of benign or malignant epithelium. This strategy is analogous to transplantation approaches used to study normal and malignant development in hematopoietic and mammary cells. The major advantage of this approach is the use of naïve adult human epithelial cells, which are the target cells for the disease. By directly transforming primary cells and transplanting them in vivo, this approach avoids potential artifacts introduced in tissue culture. While the current protocol focuses primarily on human prostate epithelium, recent literature in our lab has made use of modified versions of this protocol in both murine and human systems[2, 8-11].

Model systems of prostate cancer

Several model systems are utilized for the study of prostate cancer and the specific pathways involved. Cell lines and xenografts[12] represent the most wide-spread model in the field, and they provide valuable insight into fundamental pathways and mechanisms that support the survival and growth of prostate cancer cells. Since the majority of such cell lines are derived

from late stage or metastatic cancer, this model system is useful for uncovering and testing biochemical mechanisms *in vitro* and *in vivo*, but is not sufficient to investigate the genetic and epigenetic alterations responsible for converting healthy tissue to a malignant state. The introduction of mouse models of prostate cancer, such as prostate specific deletion of PTEN, transgenic adenocarcinoma of the prostate (TRAMP), and c-Myc, has allowed researchers to further understand the pathways leading to disease initiation and progression in an oncogene or pathway-dependent fashion[13-15]. However, development of these murine models requires complex breeding strategies and multiple crosses that are time consuming, expensive and do not allow for parallel studies with human tissue. The approach described here allows researchers to introduce oncogenes, via lentivirus, into primary prostate epithelial cells for transplantation under the kidney capsule or skin of immune-deficient mice. This technique measures both the regenerative and tumorigenic capacity of isolated prostate cell-types[9, 11]. Although the prostate-regeneration model takes cells out of their natural microenvironment and requires organogenesis concomitant with tumorigenesis, the system is faster and cheaper than mouse breeding studies, allows for efficient testing of multiple oncogene combinations, and permits parallel studies in mouse and human tissues.

Diversity of potential applications and limitations

Elucidating the pathways important for prostate stem cell function, prostate cancer initiation, and castration-resistance is critical for moving forward. Since we can now routinely isolate basal and luminal cells from benign prostates, as well as cancer cells, we can interrogate these specific cell populations using an array of genomic and proteomic approaches, including, but not limited to, microarrays, deep sequencing, activity-based protein profiling, and kinase enrichment and mass spectrometry. However, without a human prostate-regeneration system, the functional relevance of these new pathways and targets cannot be tested. Hence, the ability to consistently introduce new genes of interest, discovered using existing genomic and proteomic technologies, into primary human benign cells will shed light on new avenues and opportunities for therapeutic development.

While we are capable of utilizing many different approaches to interrogate dissociated human prostate epithelium, we are limited by the frequency at which samples are obtained and the amount of tissue that is available for processing. This technique is designed to be utilized where prostate tissue can be readily transferred to the appropriate lab quickly for processing, such as at academic institutions attached to medical centers. If prostate tissue needs to be shipped or stored for long periods of time, the cell yield and viability may be greatly reduced.

Experimental design

***In vivo* regeneration—**The subrenal regeneration assay, described in Box 4, is technically demanding and requires experience to perfect the procedure. Prior to using valuable human materials, we recommend practicing the technique with mouse prostate epithelium. If preferred, subcutaneous implantation of these cells may be performed, as described in Box 3. Using sorted cells, we have found that the rate of outgrowth formation is generally higher in the subcutaneous site compared to kidney capsule engraftment. The subcutaneous injection is an easier procedure and allows for monitoring of graft size through the skin. However, the reproducibility may be lower in the subcutaneous grafts due to the lack of blood supply, which is more abundant and consistent in the kidney capsule [16].

FACS machine accessibility—Sorting time should be arranged in advance at your institutional core facility to ensure that cells can be sorted on the same day that tissue is processed. Two types of controls are vital for accurate cell sorting, single color controls and fluorescence minus one (FMO). Control tubes should be set up such that cells are stained

with one fluorescent antibody at a time. This will allow you to determine the bleed over for each fluorophore into each channel and set the appropriate voltage compensation. FMO is a strategy to leave one fluorescent antibody out at a time to ensure that positive staining is indeed due to that specific antibody. FMO analysis should be performed when testing an antibody or setting up the gates for the first time.

Functionality of Matrigel—Lot to lot variation exists in the functional ability of Matrigel to induce sphere formation *in vitro* and/or tubule formation *in vivo*. Testing of each specific lot prior to the experiment is crucial to avoid wasting valuable sample materials. We suggest plating approximately 5,000 or 10,000 enriched prostate basal cells in Matrigel to induce sphere formation as has been previously described[10]. A good lot of Matrigel will support robust sphere formation in as little as 5 days. Growth factor reduced Matrigel has been tested briefly in the murine system with no changes in effect. However, in the human system, growth factor reduced Matrigel has not been tested.

Importance of urogenital sinus mesenchyme (UGSM)—UGSM cells are derived from the developing urogenital sinus, the site of prostate gland formation. This fetal stroma creates a highly inductive environment by secreting specific growth factors important for prostate tubule formation and development. In contrast, stromal cells isolated from the adult prostate provide growth factors that are primarily involved in maintaining tissue homeostasis. In the absence of UGSM, the prostate epithelial cells are prone to form undifferentiated cord-like structures, rather than well-differentiated tubules. Mouse UGSM is sufficient to support human prostate-regeneration, however prostate development may be enhanced with the addition of species-specific factors through the use of parallel human fetal stroma. Since human fetal stroma is difficult to obtain, we are currently testing mixtures of inductive mouse UGSM with human stromal cells, such as immortalized or primary benign and carcinoma-associated fibroblasts[7], to support human prostate epithelial growth and regeneration.

An alternative approach to using syringes for cell dissociation—The use of syringes with unscreened human material is very dangerous and represents a potential biohazard. To avoid the use of sharps during cell dissociation (see Step #12), the plunger from a 10 cc syringe can be used to mash the tissue onto either a 10 cm tissue culture dish or through a mesh strainer. Alternatively, the digested tissue can be dissociated by pipeting up and down using a plastic micro pipet tip. These alternate methods of dissociation reduce potential hazards but are less efficient at recovering epithelial cells.

Antibody dilutions—It is highly recommended to empirically determine the antibody concentration needed for efficient labeling of the cells. The dilutions outlined in this protocol are recommended as a starting point in this process.

MATERIALS

REAGENTS

- Prostate tissue: prepared from surgery through the Pathology department, as described in Box 1.
- UGSM, prepared as described in Box 2.
- NOD-SCID-IL2R$^{\gamma nul1}$ (NSG) mice Obtained from Jackson Laboratories and housed and bred under the supervision and regulations of the Division of Laboratory Animal Medicine at the University of California, Los Angeles !

CAUTION All experiments involving live rodents must conform to national and institutional standards and regulations.

- DMEM (Invitrogen, cat. no. 31800-089)
- Glutamine (Fisher Scientific, cat. no. BP379-100)
- Penicillin-Streptomycin (Omega Scientific, cat. no. PS-20)
- Collagenase, type I (Invitrogen, cat. no. 17018-029)
- Dispase (Invitrogen, cat. no. 17105-041)
- 10x PBS (Omega Scientific, cat. no. PB-10)
- Fetal bovine serum (Omega Scientific, cat. no. FB-01)
- Nu Serum (BD Biosciences, cat. no. 35504) ▲CRITICAL This serum was determined to be optimal for the growth of UGSM cells.
- Human recombinant Insulin (Invitrogen, cat. no. 12585-014)
- Fungizone Amphotericin B (Invitrogen, cat. no. 15290-018)
- RBC lysis buffer (BioLegend, cat. no. 420301)
- Trypsin/EDTA 0.05% (Invitrogen, cat. no. 25300-054)
- 10% Trypsin (Invitrogen, cat. no. 15090046)
- DNase I (Roche, cat. no. 10104159001)
- PrEGM (Clonetics, cat. no. CC-3165) complete with nine supplements to be stored at -20 °C, see REAGENT SETUP
- Matrigel (BD Biosciences, cat. no 354234) ▲CRITICAL Needs to be thawed at 4 °C according to manufacturer's specifications; each lot should be tested empirically as they may vary from lot to lot in their ability to support epithelial growth.
- Collagen (BD Biosciences, cat. no. 354236)
- 1N NaOH (Sigma, cat. no. S2770) ! CAUTION Corrosive agent; handle with care and appropriate personal protective equipment (PPE).
- CD49f antibodies: PE conjugated (eBiosciences, cat. no. 12-0495-83, clone GoH3, 0.2 mg ml^{-1}), Alexa Fluor 647 conjugated (BioLegend, cat. no. 313610, clone GoH3, concentration N/A)
- Trop2 antibodies: (R&D Systems, APC conjugated- cat. no. FAB650A, FITC conjugated- cat. no. FAB650F, PE conjugated- cat. no. FAB650P, all colors are clone 77220, 10 μg ml^{-1})
- Biotin conjugated anti-CD24 (StemCell Technologies, cat. no. 10231, clone 32D12, concentration N/A)
- APC-eFluor 780 conjugated anti CD45 (eBiosciences, cat. no. 47-0459-42, clone HI30, 3 μg ml^{-1})
- PE-Cy7 conjugated anti CD8 (eBiosciences, cat. no. 25-0088-42, clone RPA-T8, 3 μg ml^{-1})
- FITC conjugated Streptavidin (BD Biosciences, cat. no. 554060, clone N/A, 500 μg ml^{-1})

- Testosterone pellet, 12.5 mg 90 day release (Innovative Research of America, cat. no. NA-151)
- Third generation lentiviral vectors (a generous gift from Inder Verma, Salk Institute for Biological Studies, La Jolla, CA and described in more detail in Refs. [17, 18]; ref. 18 also includes a protocol for preparing and tittering lentivirus.) ! CAUTION Biosafety level II+ and appropriate PPE is required for working with lentivirus. Please conform to all national and institutional regulations prior to handling lentivirus.
- Lentiviral packaging vectors: pVSVg, pMDL, pRev (Invitrogen, cat. no. K4975-00)
- Polybrene (Hexadimethrine bromide) (Sigma, cat. no. H9268)
- Trypan blue stain 0.4% (Invitrogen, cat. no. 15250-061)

EQUIPMENT

- Tungsten Carbide Scissors 15 cm (Fine Sciences Tools)
- Ice bucket (Fisher)
- 18-, 20-, 22-G needles (Kendall)
- 10-cc syringes (BD Biosciences)
- 1-cc insulin syringes (BD Biosciences)
- Syringe attachable 22 μm pore size filters (Millipore)
- Nylon mesh filter, 40 and 100 μm pore size (BD Biosciences)
- Reichert bright line hemacytometer (Hausser Scientific)
- Alcohol and iodine prep swabs
- 6-0 Coated vicryl sutures (Ethicon)
- Wound clip applier, clips, and remover (Fisher Scientific, cat. nos. 01-804, 01-804-5, and 01-804-15, respectively)
- Cell culture centrifuge (Beckman Coulter)
- Tissue culture hood approved for use of lentivirus and human cell work (SteriGuard)
- Tissue culture water bath (Thermo Scientific)
- CO_2 incubator set to 5% or 8% CO_2 and 37°C (SteriCult)
- Adams™ Nutator Mixer (BD Biosciences)
- FACS Aria cell sorter (Becton Dickinson or similar)
- Personal protective equipment (PPE) is comprised of gloves (CURAD), an appropriate P95 or N95 Respirator (3M) and a knee length laboratory coat (Cardinal Health)

REAGENT SETUP

DMEM complete digestion solution—Add 50 ml Fetal Bovine Serum (FBS), Glutamine to a final concentration of 4 mM, Penicillin-G/Streptomycin solution (Penicillin: final concentration of 100 units ml^{-1}, Streptomycin: final concentration of 100 μg ml^{-1}), to

440 ml DMEM media. Pass media through 0.22 μm filter to sterilize. Store at 4 °C for up to 2 months.

Collagenase/Dispase digestion solution—Dissolve collagenase type I and Dispase in DMEM complete digestion solution to a final concentration of 1 mg ml^{-1} for each enzyme. Add Fungizone (final concentration of 2.5 μg ml^{-1}). Pass the media through 0.22 μm filter to sterilize. It is highly recommended to make this solution fresh each time. However, any excess can be stored at -20 °C for up to 2 weeks.

PrEGM—PrEBM basal media is supplied with 9 pre-aliquoted supplements (BPE, INSULIN, HC, GA-1000, RETINOIC ACID, TRANSFERRIN, T3, EPINEPHRINE, rhEGF). Upon receiving shipment, PrEBM media is stored at 4 °C and supplements are stored at -20 °C. Thaw 9 frozen supplements, and add all to PrEBM basal media. Filter to sterilize. Store PrEGM in dark bottle for up to 3 weeks at 4 °C or divide into 50 ml aliquots and freeze at -20 °C for long term storage of up to 2 months.

1X RBC lysis buffer—Combine 9 ml distilled water with 1 ml 10x RBC lysis buffer. Pipet to mix. Make fresh each time.

1X PBS—Dilute 50 ml 10x PBS in 450 ml distilled water and filter sterilize. Store at 25 °C for up to 12 months.

DNase I digestion solution—Dissolve 1 mg recombinant DNase I in 50 ml DMEM complete digestion solution and filter sterilize. Make fresh each time.

Collagen—Combine 250 μl collagen, 5.8 μl 1N NaOH, 28.4 μl 10x PBS. Pipet gently to mix. Storage on ice for up to 15 minutes will ensure for proper mixing and neutralization of components. Make fresh each time.

UGSM Medium—Add 25 ml FBS (5%), 25 mL NuSerum IV (5%), Glutamine to a final concentration of 4 mM, Penicillin-G/Streptomycin solution (Penicillin: final concentration of 100 units ml^{-1}, Streptomycin: final concentration of 100 μg ml^{-1}), insulin to a final concentration of 25 μl ml^{-1} to DMEM basal media. Store at 4 °C for up to 1 month.

1% Trypsin—This is for UGSM Preparation in Box 2. Dilute the 10% Trypsin (Invitrogen) to 1% (vol/vol) in sterile 1x PBS. Prepare a fresh, small aliquot each time. Do not store.

1:4 Trypan Blue Mix—Dilute 10 ml 0.4% Trypan Blue into 30 ml 1x PBS for a total of 40 ml.

PROCEDURE

Weighing and preparing tissue for overnight enzymatic digestion ● TIMING 1 h

1 Obtain tissue samples from Pathology (Fig. 1a). Trim marked regions of tissue using scissors (Fig. 1b), and measure the weight of unmarked tissue in grams (Fig. 1c). Transfer tissue to a 100 mm × 20 mm tissue culture dish (Fig. 1d).

! CAUTION All work with primary human tissue needs to be done in an approved tissue culture hood with proper attire including PPE.

2 Use scissors to cut tissue into small chunks. Add a small amount of PBS to keep tissue together and continue to cut until chunks are small enough to pass through

a 5 ml pipet. Transfer tissue chunks/PBS to 50 ml conical tube using a 5 ml pipet and wash the tissue culture dish with additional PBS as needed.

3 Spin down tissue at 1800 rpm (754 xg) for 5 min at 4 °C. Aspirate supernatant and resuspend pellet in 30 ml PBS. Repeat spin to wash.

4 Aspirate supernatant and resuspend pellet in 20-30 ml DMEM complete digestion solution. Spin down tissue at 1800 rpm (754 xg) for 5 min at 4 °C.

5 While centrifuging, prepare collagenase/dispase digestion solution (See reagent setup). Calculate total volume needed (10 ml per gram of tissue and up to 50 ml for 5 grams in a single 50 ml conical tube).

6 Aspirate supernatant from tissue in step 4 and resuspend in collagenase/dispase digestion solution. For greater than 5 grams of starting tissue, the digestion process will need to be carried out in multiple 50 ml conical tubes. ■ PAUSE POINT If samples are processed earlier in the day, then tissue can be kept on ice or at 4 °C until the end of the day in DMEM complete digestion solution, prior to overnight digestion at 37 °C in collagenase/dispase digestion solution.

Overnight enzymatic digestion of tissue ● TIMING 8-12 h

7 Digest tissue chunks in 37 °C incubator overnight with rocking action on an Adams™ Nutator Mixer. ▲CRITICAL STEP Length of digestion depends on size of tissue sample and timing of tissue processing. Overnight digestion has been optimized for tissue that is processed at the end of the day, for samples that weigh approximately 1-5 grams. For smaller samples, digestion time should be decreased.

Preparation of single cells ● TIMING 1-2 h

8 The next morning, spin down cells/digestion mix at 1800 rpm (754 xg) for 5 min at 4 °C.

? TROUBLESHOOTING

9 Aspirate the supernatant, wash cells 2 times in 25 ml PBS and spin down at 1800 rpm (754 xg) for 5 min at 4 °C.

10 Aspirate supernatant, resuspend cells in 5 ml 0.05% Trypsin/EDTA, incubate for 5 min in a 37 °C degree water bath with occasional shaking to ensure that the enzyme can access all of the tissue.

11 During incubation, prepare DNase I digestion solution. After 5 minutes in water bath, add 15 ml DNase I digestion solution to trypsinized cells and mix. Spin down cells at 1800 rpm (754 xg) for 5 min at 4 °C. ▲CRITICAL STEP In the absence of DNase I, the trypsinized tissue will stick together and make it very difficult to go through the syringe in step 12.

12 Aspirate supernatant, resuspend in 10 ml DMEM complete digestion solution. Draw cells/media up and down through 18 gauge needle attached to a 10 cc syringe up to 5 times. Repeat with 20 and 22 gauge needles if possible. ! CAUTION The use of sharps around unscreened human biological material is very dangerous. Please take extra care when using syringes in this manner. An alternative approach to using sharps is discussed in the experimental design section of the introduction.

? TROUBLESHOOTING

13 Filter cells/media through 100 μm cell strainer and wash the conical tube and filter with an additional 10 ml DMEM complete digestion solution. Spin down cells at 1800 rpm (754 xg) for 5 min at 4 °C.

? TROUBLESHOOTING

14 During spin, prepare 1x RBC lysis buffer (See reagent setup).

15 Aspirate supernatant and resuspend cell pellet in 5 ml 1x RBC lysis buffer. Keep cells/lysis buffer on ice for 5 minutes with occasional shaking. After 5 minutes, add 25 ml PBS to conical tube and spin down cells at 1800 rpm (754 xg) for 5 min at 4 °C. ▲CRITICAL STEP If collection of red blood cells is desired, step 15 should be avoided. In the absence of RBC lysis, cell sorting time may be significantly increased due to the large number of red blood cells (Fig. 1e).

16 Aspirate supernatant and resuspend cell pellet in 10 ml DMEM complete digestion solution. Filter cells through 40 μm cell strainer. Wash the tube and strainer with an additional 10 ml DMEM complete digestion solution. Spin down cells at 1800 rpm (754 xg) for 5 min at 4 °C.

17 Aspirate supernatant and resuspend cell pellet in 1-3 ml PrEGM complete media. Count cells by hemacytometer to determine the yield (Fig. 1f). Approximately 5 million total cells can regularly be collected per gram of starting tissue. ■ PAUSE POINT Dissociated cells can be stored on ice or at 4 °C for several hours prior to sorting, depending on the availability of the FACS machine.

? TROUBLESHOOTING

Staining cells for FACS ● TIMING 1 h

18 Label appropriate number of FACS tubes (1 tube for unstained cells, 1 separate tube per single fluorescent antibody control, 1 tube for “all” antibodies)

19 Aliquot approximately 1-5 × 10^4 cells in 100 μl PrEGM media per tube for unstained control and single antibody controls, used to set the compensation for sorting experiments. Add the appropriate amount of antibody as indicated in Table 1 for single antibody controls.

20 Prepare remaining cells in the tube labeled “all” at a concentration of no more than 1 × 10^7 cells per ml of PrEGM. Add appropriate amounts of antibody as indicated in Table 1 for sorted sample.

21 Incubate tubes on ice with a cover to block light for 30 min with occasional shaking. ▲CRITICAL STEP Conjugated fluorophores are sensitive to ambient light. Mixing is essential to ensure exposure of antibody to all cells.

22 Spin down cells at 1800 rpm (754 xg) for 5 min at 4 °C.

23 Aspirate supernatant, resuspend in 1 ml PrEGM media to wash, and spin down cells at 1800 rpm (754 xg) for 5 min at 4 °C.

24 If secondary antibody (FITC conjugated streptavidin) is required, resuspend cells used for single antibody control in 100 μl PrEGM, and resuspend cells used for “all” in the appropriate volume (no more than 1 × 10^7 cells per ml) of PrEGM. If secondary antibody is not required, resuspend single color antibody samples in 300 μl PrEGM and keep on ice.

25 Incubate cells with secondary antibody on ice with a cover for 15 min with occasional shaking. ▲CRITICAL STEP Conjugated fluorophores are sensitive to ambient light. Mixing is essential to ensure exposure of antibody to all cells.

26 Spin down cells at 1800 rpm (754 xg) for 5 min at 4 °C.

27 Aspirate supernatant, resuspend cells in 1 ml PrEGM media to wash, spin down cells at 1800 rpm (754 xg) for 5 min at 4 °C.

28 Aspirate supernatant, resuspend single antibody control samples in 300 μl PrEGM. Resuspend "all" sample in 1-2 ml PrEGM. Filter "all" sample through a 40 μm cell strainer. Wash tube and strainer with an additional 1 ml PrEGM. Combine 2-3 ml of cells/PrEGM, transfer to a new FACS tube for sorting. Keep all tubes on ice. ▲CRITICAL STEP Cells tend to clump after spinning down and can clog the fluidics of the cell sorter without filtering the sample first.

Isolation of distinct epithelial and non-epithelial cell subsets by FACS ● TIMING 2-4 h

29 Run individual antibody controls and unstained cells on FACSAria II to set the correct compensation and voltage for sorting. Set FACS gates as indicated in Figure 2, and collect up to four distinct cell populations simultaneously. If greater than four populations are desired, switching of collection tubes between samples is required. Immune cells are marked by expression of the pan-leukocyte marker CD45 and can be subdivided into $CD45^+CD8^+$ lymphocytes and the remaining $CD45^+CD8^-$ immune cells. $Trop2^+CD45^-$ epithelial cells can be further subdivided into basal ($Trop2^+CD49f^{hi}CD24^{lo/+}$) and luminal ($Trop2^+CD49f^{lo}CD24^+$) subsets (Fig 2). Collect cells in 1 ml of 50% PrEGM/ 50% FBS. ! CAUTION Sorting can create an aerosol. All sorting of human cells should be done with a vacuum.

30 Spin down cells at 1800 rpm (754 xg) for 5 min at 4 °C. Aspirate supernatant and resuspend in 1 ml PrEGM. ■ PAUSE POINT Sorted cells can be stored on ice prior to counting and infection. For other types of analysis including protein, RNA or DNA, sorted cell pellets or lysates can be stored at -80 °C long-term.

31 Mix 10 μl cells/PrEGM with 10 μl 1:4 Trypan Blue Mix, and count the sorted prostate epithelial cells by hemacytometer. Viable healthy cells exclude the blue dye. This assay can be used to determine the degree of viability in a sample. Set aside a subset of sorted cells as the control sample for the transduction efficiency assay described in Box 5.

Lentivirus infection ●TIMING 1 h

! CAUTION Lentivirus is highly infectious and the use of third generation lentiviral vectors is highly recommended (Fig. 3). All lentivirus is pseudotyped with VSV-g for efficient transduction using either mouse or human cells.

32 Pipet the appropriate number of cells (5×10^4- 5×10^5 per condition) into polystyrene FACS tubes depending on the number of grafts or unique genes you want to over-express. Usually a target cell number of 5×10^4 - 1×10^5 cells per graft are desirable. For multiple grafts, combining up to 5×10^5 cells in a single tube is sufficient. Total volume should be between 100 – 500 μl per tube.

33 Add Polybrene to each tube at a final concentration of 8 μg/ml (include the amount needed for lentivirus into the calculated total volume).

34 Add the appropriate high-titer lentivirus to the tube in a typical multiplicity of infection (MOI) in the range of 25-75, as determined by infection on 293T cells[18]. ! CAUTION All experiments involving lentivirus must conform to institutional regulations and appropriate PPE must be worn at all times.

35 Place the tubes at 37 °C in a sterile tissue culture incubator, designated for lentivirus only, for 1 hour making sure to mix the tubes every 15 minutes by flicking.

Lentivirus spinfection, washing and preparation of grafts ● TIMING 1.5 h

36 After 1 hour, centrifuge the tubes at 1800 rpm (754 xg) for 1 hour at 25 °C. Wash the tubes 3 times with PBS to remove any unbound lentivirus. Set aside a subset of transduced cells as the test sample for the transduction efficiency assay described in Box 5.

37 Combine cells with equal numbers of cultured Urogenital Sinus Mesenchyme (UGSM) cells. See Box 1 and ref. 10 for UGSM isolation, expansion and preparation. Spin down cells at 1800 rpm (754 xg) for 5 min at 4 °C. ▲CRITICAL STEP UGSM cells are essential for supporting the regenerative capacity of primary epithelium.

38 Aspirate supernatant, place cell pellet on ice. To prepare Matrigel grafts, follow option A. Prepare collagen grafts according to option B. ■ PAUSE POINT Grafts can be kept in a standard tissue culture incubator at 37 °C overnight prior to surgery the following day. After 24 hours, UGSM cells start to migrate out of grafts and collagen grafts can come apart.

Option A : Preparing Matrigel grafts

i Resuspend cell pellet in approximately 30 µl per graft, transfer to an eppendorf tube and keep on ice.

▲CRITICAL STEP Thaw Matrigel on ice (may take 1-2 hours) and ensure that this step is carried out on ice, as Matrigel can solidify quickly at warmer temperatures. Pipet carefully and avoid bubbles when resuspending cell pellets.

▲CRITICAL STEP To prepare multiple Matrigel grafts using cells from the same condition, aliquot 30 µl of Matrigel/cells per replicate into individual eppendorf tubes.

Option B: Preparing collagen grafts

i Resuspend cell pellet in approximately 15 µl per graft.

▲CRITICAL STEP Ensure that this step is carried out on ice, as collagen can solidify quickly at warmer temperatures. Pipet carefully and avoid bubbles when resuspending cell pellets.

ii Pipet 15 µl of cells/collagen mixture onto tissue culture dish and incubate 20 min at 37 °C to solidify.

iii Add UGSM media to the dish to cover grafts prior to implantation into mice.

▲CRITICAL STEP If multiple collagen grafts are desired, prepare a master mix of cells/collagen and pipet 15 µl per graft onto separate wells of a 6-well tissue culture dish and place the grafts at 37 °C for 20-30 minutes to allow them to solidify. Multiple grafts of the same type can be placed into a single well.

39 In vivo transplantation options are described in Box 3 for subcutaneous injection and Box 4 for subrenal (kidney capsule) regeneration. Harvest grafts after 8-16 weeks in vivo and fix tissue using standard techniques for fixation. ! CAUTION All experiments involving live rodents must conform to national and institutional regulations.

? TROUBLESHOOTING

Steps 1-6, Weighing and preparing tissue for overnight enzymatic digestion: 1 h

Step 7, Overnight enzymatic digestion of tissue: 8-12 h

Steps 8-17, Preparation of single cells: 1-2 h

Steps 18-28, Staining cells for FACS: 1 h

Steps 29-31, Isolation of distinct epithelial and non-epithelial cell subsets by FACS: 2-4 h

Steps 32-35, Lentivirus infection: 1 h

Steps 36-39, Lentivirus spinfection, washing and preparation of grafts: 1.5 h

TROUBLESHOOTING

Troubleshooting advice is provided in Table 2.

ANTICIPATED RESULTS

Normal prostate regeneration (Fig. 4a) or transformation (Fig. 4b) can be analyzed by staining paraffin-embedded tissue with Haematoxylin & Eosin (H&E) and human prostate luminal cell markers such as prostate-specific antigen (PSA). Normal-regenerated tubules (Fig. 4c) contain distinct layers of basal cells (p63+, AR-low) and luminal cells (p63-, AR+). Prostate tubule regeneration is a property of basal stem-like cells, purified based on the antigenic profile $CD45^{-}Trop2^{+}CD49f^{hi}$. Total prostate cells or total epithelial cells can also be used as the source of regenerative cells, however purifying the basal fraction allows for the greatest regenerative capacity. As few as 5000 basal cells are sufficient to regenerate prostatic tubules. We have reported that introduction of two (AKT, ERG) or three (AKT, ERG, AR) oncogenes can transform cells from the basal fraction[2]. While basal cells are an efficient target cell for transformation, additional oncogenic influences may be capable of transforming other cell populations. We have previously demonstrated that both basal and luminal cells can be transduced with lentivirus, and that the resulting structures retain expression of both fluorescent markers and transgenes[2]. To ensure that target cells are infected with lentivirus, a subset of infected cells and control non-transduced cells can be left in culture for several days to look for evidence of fluorescent marker and transgene expression, as indicated in Box 5 in the transduction efficiency assay. A typical experiment will yield approximately 30-40% transduced cells.

Box 1. Preparation of tissue from robotic radical prostatectomy surgery and Pathology
Following Institutional Review Board Approval, patients scheduled to undergo radical prostatectomy [robot-assisted laparoscopic radical prostatectomy (RALP) or open radical retropubic prostatectomy (RRP)] are consented for research participation to collect blood and prostate tissue. After an overnight fast, patients are brought to the operating room where general anesthesia is induced and a standard RALP or RRP is performed. The Pathology department is contacted just prior to removal of the specimen to facilitate immediate transportation of fresh tissue to Pathology for subsequent analyses.

▲CRITICAL STEP While every attempt is made to standardize the surgical procedure, it should be noted that differences in surgeon, surgical technique, intra-operative variables, and operative time may all affect the time at which the specimen is sent for analysis. As a result, tissue hypoxia times may vary considerably between specimens.

The prostate is transported to surgical pathology at room temperature (25 °C) as soon as it is removed from the patient. A trained and licensed pathology assistant weighs and measures the prostate, inks its surface with Indian ink (to assess the status of surgical margins on histologic sections) and slices the prostate from base to apex. Under an IRB-approved protocol, slices 2 and 4 of the prostate are used for research while the rest is entirely submitted for pathologic diagnosis. Slices 2 and 4 are divided into 4 quadrants and additional ink of different colors is used for orientation indicating right vs. left, anterior vs. posterior. The procured tissue is separated into top and bottom portions with a knife, keeping enough tissue on the top for frozen section diagnosis while preserving as much fresh tissue in the bottom as possible. Tissue from the top is snap-frozen and a frozen section slide is prepared and stained with a hematoxylin and eosin (H&E)-based protocol. The pathologist examines the frozen section slides under the microscope and circles the cancer and benign areas. The fresh tissue (bottom) is then matched with the frozen section slides and the cancer and benign areas are separated manually. Care is taken to ensure that benign regions are free of cancer, however cancer regions will regularly contain surrounding benign areas.

Box 2. Urogenital Sinus Mesenchyme (UGSM) Preparation

UGSM cells are essential for proper in vivo regeneration. For the sake of completeness and ease of implementing the procedure, the information provided here has been reproduced and adapted from a prior protocol where we described the preparation of UGSM cells[10].

1. Set up matings for timed pregnancies. Sacrifice pregnant female mice at E16 (embryonic day 16 of pregnancy.)
2. Take the uterus with the embryos, and move to a 10 cm dish containing DMEM complete digestion media. Cut the uterus laterally, separate embryos from the placenta, and place in a fresh dish containing DMEM complete digestion media.
3. Cut embryos in half, below the liver. Place the bottom half of the embryos into a new dish containing sterile 1x PBS. Place the bottom half of the embryos in a supine position and cut the abdomen open while holding the hind legs apart with forceps.
4. The urogenital sinus is connected to the bladder. As in the adult, the urogenital sinus could be removed intact by gently pulling up on the bladder. Dissect the pelvic UGS, clean off the attached tubular structures, and cut off the bladder.
5. Place the pelvic UGS onto a concave glass slide containing 250 μl DMEM complete digestion media. When each UGS has been collected, wash all tissues 3 times with 1x PBS. Aspirate the last wash of PBS carefully, and add 1 ml 1% Trypsin. Keep the plate in 4 °C for 90 minutes to allow for digestion.
6. Carefully remove the Trypsin with a pipette, and add DMEM complete digestion media. Carefully pipette the media off, and add 1 ml DMEM complete digestion media containing 500U DNase I. Let sit for 5 minutes.
7. Wash the UGS three more times with fresh DMEM complete digestion media.

8. After the third wash, take 2 28-gauge needles and separate the mesenchyme away from the epithelium. The epithelium can be identified as the opaque cylinder shaped object inside the more translucent and vascular mesenchyme.
9. Collect all of the mesenchyme fragments into a 15 ml Falcon tube containing 10 ml Collagenase/Dispase digestion solution. Digest at 37 °C for 2 hours with rocking action on an Adams™ Nutator Mixer.
10. After digestion, filter the cells through a nylon mesh filter with 40 μm pore size, and wash the filter with 10 ml DMEM complete digestion media. Spin down cells at 1300 rpm (400 xg) at 25 °C for 5 minutes. Aspirate media, resuspend cells in 10 ml UGSM Media. Plate in a 10 cm tissue culture dish.
11. Culture for 5–7 days, and monitor cell growth. Passage and expand the UGSM cells when they get to 80% confluency. ■ PAUSE POINT UGSM cells can be frozen down and stored at −80 °C or in liquid nitrogen for up to 6 months. Do not passage UGSM cells more than 5 times, as they lose their inductive capacity in later passages.

Box 3. Subcutaneous injection

For the sake of completeness and ease of implementing the procedure, the information provided here has been reproduced and adapted from a prior protocol where we described subcutaneous injection of primary epithelial cell grafts for in vivo regeneration[10].

1. Anesthetize a male immune-deficient mouse with the appropriate anesthetic such as Ketamine/Xylazine or Isofluorine gas approved by national and institutional regulations. Once the animal is anesthetized, place the mouse prone and shave the back of the mouse. Sterilize the target injection site by alternating iodine and alcohol prep swabs 3 times.
2. Immediately before injection, gently pipet to mix the 30 μl cell mixture in eppendorf tube on ice. Draw up the mixture into an insulin syringe, making sure to avoid bubbles.
3. Using a clean pair of forceps, pull up on the freshly shaved and cleaned skin of the mouse, creating a tent between the skin and musculature of the flank. Inject the mixture, and pull out the needle slowly. Hold onto the skin with the forceps for a minute to make sure the Matrigel has settled.
4. Implant a testosterone pellet subcutaneously in mice to ensure an environment with excess androgen. This step requires making a small incision through the outer layer of skin, implanting the pellet, and using metal clips to staple the wound in the skin.
5. Monitor and medicate the mice in accordance to national and institution animal care protocols. Remove staples 7 days after procedure.

Box 4. Subrenal regeneration assay

For the sake of completeness and ease of implementing the procedure, the information provided here has been reproduced and adapted from a prior protocol where we described implanting primary epithelial cell grafts under the kidney capsule for in vivo regeneration[10].

1. Anesthetize a male immune-deficient mouse with the appropriate anesthetic suc as Ketamine/Xylazine or Isofluorine gas approved by national and institutional

regulations. Once the animal is anesthetized, place the mouse prone, and shave a 3cm × 3cm area in the mid back region. Sterilize the surgical area by alternating iodine and alcohol prep swabs 3 times.

2. Cut a small incision in the skin around the middle of the back, approximately 1cm lateral to the spine. Cut a second small hole in the peritoneum exposed.
3. Hold the peritoneum open using forceps, and search for the kidney. Once located, use the small fat pad located at the tip of the kidney to pull it to the surface, and out above the skin.
4. Gently grab the thin membrane on the outside of the kidney with two forceps, and tear a small hole. Lift the edge of the capsule; push the collagen graft underneath the membrane. 1 to 4 collagen grafts can be inserted per kidney capsule depending on the size of the kidney. ▲CRITICAL STEP Make sure the collagen plug is securely underneath the kidney capsule.
5. Grab the peritoneum with forceps, and gently allow the kidney to slip back into the body. Make sure the graft doesn't slip out. Suture the peritoneum together, careful not to puncture any organs. Insert a testosterone pellet subcutaneously. Finally, use metal clips to staple the skin closed.
6. Monitor and medicate the mice in accordance to national and institutional animal care protocols. Remove staples 7 days after surgery.

Box 5. Transduction efficiency assay

1. Take a small aliquot of primary prostate epithelial cells that have been infected with lentivirus and washed three times (from step 36), and an aliquot of cells that have not been exposed to lentivirus as a control (from step 31). Approximately 1×10^4 - 1×10^5 cells can be evaluated in this assay. Keep transduced and control cells separate at all times. Transfer cells into an eppendorf tube in 100 µl PrEGM. Add 100 µl of pre-thawed Matrigel and mix gently by pipetting up and down.
2. Plate 200 µl mixture into the center of a single well in a 12-well tissue culture dish. Transfer the dish to a tissue culture incubator set at 37 °C and 5-8% CO_2 to allow mixture to solidify. This step takes approximately 30-45 minutes.
3. When the mixture has solidified, add 1 ml PrEGM to each well.
4. Return dish to incubator and allow cells to grow and take up lentivirus for 72 hours until the fluorescent color marker from the lentivirus is expressed at detectable levels in transduced cells.
5. Remove media from each well taking care not to disturb the Matrigel. Add 1 ml Collagenase/Dispase Digestion Solution to each well and return dish to the incubator for 1 hour.
6. At this point, Matrigel will be disrupted and cells should be floating in each well. Collect cells into a FACS tube. Add additional PrEGM to the tube. Centrifuge the tubes at 1800 rpm (754 xg) for 5 minutes at room temperature.
7. Resuspend cells in 300 µl PrEGM.
8. Run samples on flow cytometer. Use the control tube to set the positive fluorescence gate. 0% of control cells should appear in the positive gate. Run the transduced cell samples. The percentage of cells in the positive gate

represents the percentage of cells expressing detectable levels of fluorescence from the lentiviral cassette.

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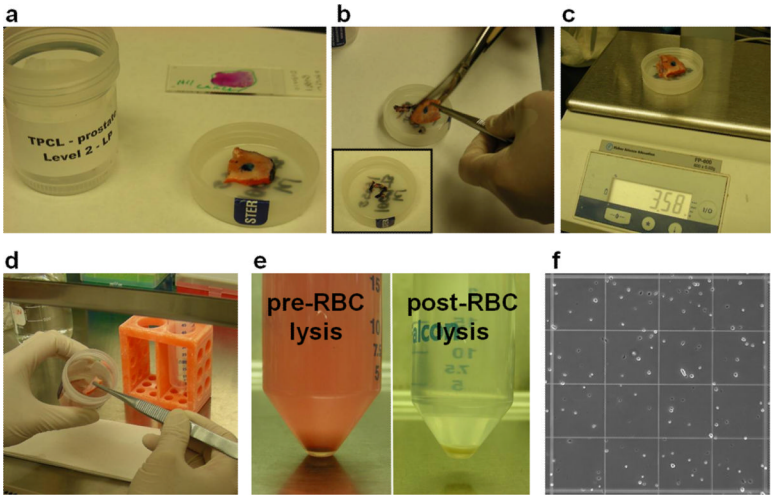

Figure 1. Tissue preparation to single cells

(a) Tissue is marked up by the Pathology core laboratories. (b) Marked edges of the tissue are removed manually using scissors (inset shows trimmed edges), (c) and the remaining unmarked tissue is weighed. (d) Tissue is transferred to a tissue culture dish to be cut into small fragments. (e) After overnight digestion, samples contain red blood cells (left) that can be lysed to leave remaining cells (right). (f) Single dissociated cells can be counted by hemacytometer.

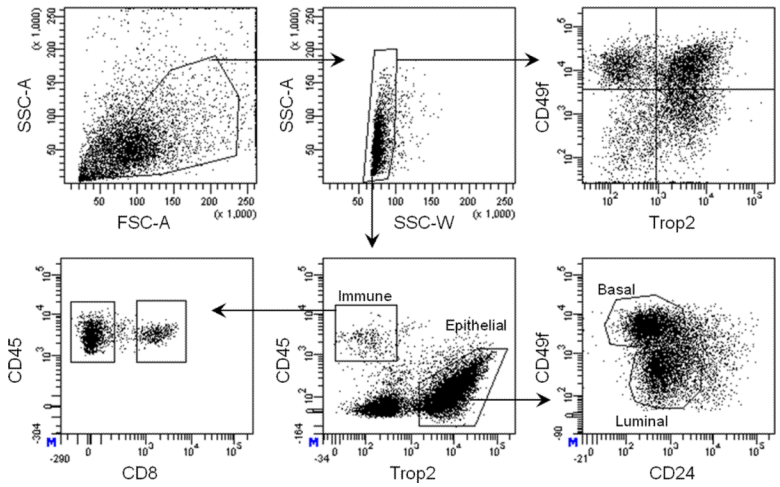

Figure 2. Gating strategy to purify basal, luminal and immune cell subsets

FACS plots demonstrate the gating strategy used to isolate total cells based on forward scatter (FSC) and side scatter (SSC), and to exclude doublets using SSC width vs. area. Total cells can be analyzed by expression of CD49f and Trop2, as has been previously reported. A more complex sorting strategy includes separation of immune cells ($CD45^{+}$) and epithelial cells ($Trop2^{+}$). The CD45+ fraction is comprised of both CD8+ lymphocytes and CD8- immune cells, while the Trop2+ epithelium can be subdivided into basal ($Trop2^{+}CD49f^{hi}CD24^{lo/+}$) and luminal ($Trop2^{+}CD49f^{lo}CD24^{+}$) fractions.

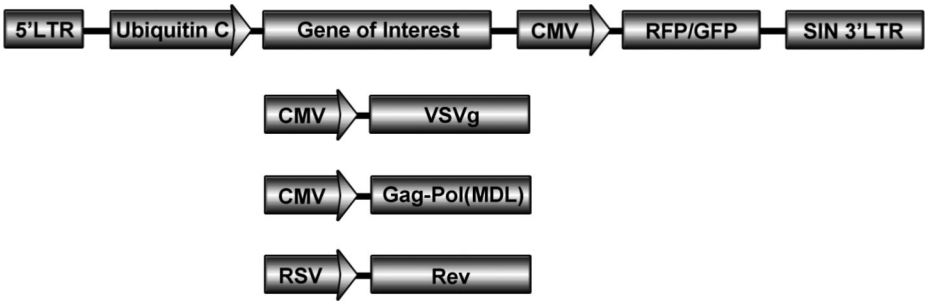

Figure 3. Schematic of third generation lentiviral and packaging vectors

The lentiviral vector (top) expresses your gene of interest along with a fluorescent color marker such as GFP or RFP for titering and expression *in vivo*. The packaging vectors (pVSVg, pMDL, pRev) are necessary for proper assembly of the lentivirus production. Please see Tiscornia, *et al.* (2006) for more detailed information on this system and how to efficiently produce high titer lentivirus[18].

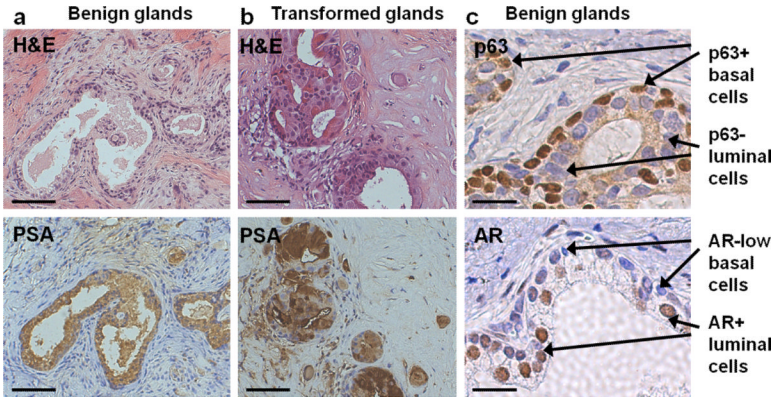

Figure 4. Regeneration of benign and transformed human prostate glands in vivo

(a) Basal cells without genetic manipulation generate phenotypically benign glands with expression of prostate-specific antigen (PSA) indicating differentiation to the luminal lineage. Scale bars, 100 μm. (b) Basal cells manipulated to express selected oncogenes can generate malignant lesions, characterized by increased staining for PSA and loss of basal cells. Scale bars, 100 μm. (c) High-power images of benign tissue stained for the transcription factors p63 and AR reveal the presence of distinct layers of p63+ AR-low basal cells and p63- AR+ luminal cells. Scale bars, 25 μm.

Table 1

Antibodies used to identify and isolate distinct epithelial and immune cell subsets

Antigen	Conjugate (Company)	Volume (μl) for Single Antibody Controls (1 × 10E4 cells)	Volume (μl) for Sorted Sample (per 1 × 10E6 cells/100 (μl)
CD49f	PE (eBiosciences)	1	3
	Alexa647 (BioLegend)	1	3
Trop2	APC (R&D)	3	10
CD24	Biotin (StemCell Technologies)	5	15
Streptavidin	FITC (BD)	1	5
CD45	APC-eFluor780 (eBiosciences)	1	5
CD8	PE-cy7 (eBiosciences)	1	5

Table 2

Troubleshooting Table

Step	Problem	Possible Reason	Solution
8	Incomplete pelleting of entire sample.	Residual fat tissue is present and will not spin down.	Increase spin speed and/or time to pellet cells. Addition of FBS has been shown to increase pelleting.
12	Sample will not easily be drawn through the syringe.	Cells can be clumpy and/or stringy due to incomplete digestion or dead cell debris clumping together.	For clumping, add an additional 5 minute digest in trypsin. For stringy cell debris, incubate the cells with DNase I solution for longer time points, and pipet/plunge with force against the bottom of a tissue culture dish to break up into smaller pieces.
13	Sample does not filter easily.	The amount of tissue exceeds the capacity of the filter and blocks the flow of sample through it.	Use multiple filters or a filter with a larger surface area. Alternatively, flushing the sample through the filter at an increased rate will reduce the amount of filters needed.
17	Low cell yield.	Increased number of dead cells due to lengthy digest of a small sample.	Reduce the length of collagenase/dispase digestion with smaller samples. See step 7 for more details.
39	Poor regeneration	Disparity in health of patient cells, supportive nature of UGSM due to passage, and lot to lot variation of Matrigel.	Do not use patient cells for *in vivo* experiments if trypan blue staining post sort is high. Keep UGSM passage low. Ensure that Matrigel can support cell growth *in vitro* before using *in vivo*.

PC3 Is a Cell Line Characteristic of Prostatic Small Cell Carcinoma

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BACKGROUND. The majority of the prostatic cancers are adenocarcinomas characterized by glandular formation and the expression of luminal differentiation markers androgen receptor (AR) and prostate-specific antigen (PSA). Most adenocarcinomas are indolent and androgen-dependent. Hormonal therapy that inhibits AR signaling produces symptomatic relief in patients with advanced and metastatic adenocarcinomas. Prostatic small cell neuroendocrine carcinoma (SCNC) is a variant form of prostate cancer (PC). In contrast to adenocarcinoma, the tumor cells of SCNC do not form glands and are negative for AR and PSA. SCNC is extremely aggressive and does not respond to hormonal therapy. The purpose of this study was to compare the important and relevant features of two most commonly used PC cell lines, LNCaP and PC3, with prostatic adenocarcinoma and SCNC.
METHODS. Xenograft tumors of LNCaP and PC3 were prepared and compared with human prostatic adenocarcinoma and SCNC for the expression of key signaling molecules by immunohistochemistry and Western blot analysis.
RESULTS. LNCaP cells express AR and PSA and their growth is inhibited by androgen withdrawal, similar to human prostatic adenocarcinoma. PC3 cells do not express AR and PSA and their proliferation is independent of androgen, similar to SCNC. Adenocarcinoma cells and LNCaP cells are negative for neuroendocrine markers and stem cell-associated marker CD44 while SCNC and PC3 cells are positive. LNCaP cells have identical cytokeratin profiles to adenocarcinoma while PC3 cells have cytokeratin profiles similar to SCNC.
CONCLUSION. LNCaP cells share common features with adenocarcinoma while PC3 cells are characteristic of SCNC. *Prostate 71: 1668–1679, 2011.* © 2011 Wiley-Liss, Inc.

KEY WORDS: **prostate cancer; small cell carcinoma; adenocarcinoma; PC3; LNCaP**

Additional Supporting Information may be found in the online version of this article.

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INTRODUCTION

Prostate cancer (PC) is the most common malignancy in men and the second leading cause of cancer-related deaths [1]. Normal prostate epithelium contains luminal epithelial cells, basal cells and a small component of neuroendocrine (NE) cells that are scattered throughout the prostate [2–5]. The majority of the PCs are classified as adenocarcinomas characterized by an absence of basal cells and uncontrolled proliferation of malignant tumor cells with features of luminal differentiation including glandular formation and the expression of androgen receptor (AR) and prostate-specific antigen (PSA). Interestingly, every single case of prostatic adenocarcinoma also contains a small population (usually ~1%) of NE tumor cells [2–5]. The NE cells in adenocarcinoma share many important features with those in the benign prostate. For example, in contrast to the non-NE luminal-type tumor cells, the NE cells in benign prostate and adenocarcinoma do not express AR and PSA [6,7].

A minority of the prostatic epithelial malignancies are variant forms including ductal type adenocarcinoma, mucinous (colloid) carcinoma, signet ring cell carcinoma, and small cell (neuroendocrine) carcinoma (SCNC) [8]. Prostatic SCNCs are considered indistinguishable from pulmonary and other extra-pulmonary SCNCs with a solid, sheet-like growth pattern, usually with areas of tumor necrosis. Tumor cells are small, with fine chromatin pattern, scant cytoplasm, and nuclear molding. Mitotic figures and crush artifact are frequent findings [3,9,10]. SCNCs of the prostate are rare tumors and account for no more than 1% of all carcinomas of the prostate. Although they may arise de novo, such tumors are often seen as recurrent tumors in patients who have a history of conventional prostatic adenocarcinomas and received hormonal therapy [11,12]. SCNC may be present either as a pure form or as a component of mixed tumors which also contain conventional adenocarcinoma. Similar to the NE cells in benign prostate and prostatic adenocarcinoma, the tumor cells in SCNC lack the expression of AR and PSA [9,10,13], which explains the clinical observation that such tumors, unlike adenocarcinomas, do not respond to hormonal therapy that stops androgen production and inhibits AR function [14,15]. In contrast to the majority of prostatic adenocarcinomas that pursue an indolent clinical course, SCNC is highly aggressive, usually presenting with locally advanced disease or distant metastasis, and the patients usually die within months of the diagnosis [16,17]. Therefore, SCNC is a different tumor than prostatic adenocarcinoma and the two entities should be clearly distinguished.

Because PC is a highly prevalent disease, it has been the focus of significant research activities for many years. Numerous articles have been published studying PC using various models including established cell lines derived from metastatic human PCs, xenograft models, and genetically engineered mouse models of PC. Among these models, cell lines have had the longest history and been most widely used in publications. Two of the most commonly used cell lines are LNCaP [18,19] and PC3 cells [20], derived from lymph node and bone metastases, respectively. It has been well established through numerous studies that LNCaP cells express AR and PSA, are androgen-dependent with relatively indolent biologic behavior similar to the vast majority of the PCs encountered clinically [19,21]. PC3 cells, on the other hand, do not express AR and PSA and are androgen-independent [20–21]. They show highly aggressive behavior which is unlike most clinical cases of PCs. For many years, these two cell lines have been used by researchers to represent different spectrums of PC with LNCaP as the indolent form and PC3 as the aggressive form of PC. They have also been used to represent androgen-dependent and castration-resistant tumors, respectively. Here we present evidence showing that unlike LNCaP cells which are typical of conventional adenocarcinoma of the prostate, PC3 cells have features that are characteristic of prostatic SCNCs.

MATERIALS AND METHODS

Materials

PC-3 and LNCaP cells were obtained from the American Type culture Collection (ATCC); fetal bovine serum (FBS), RPMI medium 1640, Dulbecco's modified Eagle's medium (DMEM), sodium pyruvate, L-glutamine, penicillin, and streptomycin were purchased from Hyclone; charcoal/dextran-treated FBS medium was purchased from Cellgro; monoclonal anti-neuron-specific enolase (NSE) antibody was from DAKO (Carpinteria, CA); monoclonal anti-chromogranin A (CgA) was from Neomarkers (Fremont, CA); polyclonal anti-PSA antibody, polyclonal anti-AR antibody, and monoclonal anti-β-actin antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-CD44 antibody was from eBiosciences (San Diego, CA). Monoclonal anti-P53 antibody was from EMD (San Diego, CA). Polyclonal anti-CK5 and monoclonal anti-CK8 antibodies were from Covance (Emeryville, CA).

Cell Culture

LNCaP cells were maintained in RPMI 1640 supplemented with 10% FBS and penicillin–streptomycin,

whereas PC-3 cells were maintained in the DMEM containing 10% FBS, penicillin–streptomycin, L-glutamine, pyruvate sodium. Cells were grown at 37°C with 5% CO_2.

Growth Curve

Equal numbers of cells were seeded into 96-well plates and maintained with the normal medium supplemented with regular FBS or charcoal-stripped FBS (androgen-deprived). Growth curves were obtained by the Promega CellTiter (Madison) assay according to manufacturer's instructions on days 1, 3, 5, and 7. The experiments were performed in quadruplicates.

Xenograft Tumors and HumanTumors

LNCaP and PC3 cells were used to generate xenograft tumors in male severe combined immunodeficient (SCID) mice as described [22]. When the cells reached ~80% confluency, they were washed with cold PBS and trypsinized. The cells were washed once in media and resuspended to a concentration of 1×10^6 cells/50 μl (PC3) or 2×10^6 cells/50 μl and mixed with an equal volume of cultrex (R&D Systems). The mixture was then injected into the flanks of immunodeficient mice for tumor development. The tumors were harvested, fixed in formalin, and embedded in paraffin. Paraffin sections were prepared for immunohistochemical studies.

For human adenocarcinoma, we used a tissue microarray containing tissue cores from 80 cases as published previously [23]. Regular sections from five cases of the small cell carcinoma cases were used as reported in a previous publication [24].

Immunohistochemistry

The detailed method has been reported previously [23]. Briefly, paraffin sections of 4-μm thickness were deparafinized with xylene and rehydrated through graded ethanol. Endogenous peroxidase activity was blocked with 3% hydrogen perozide in methanol for 10 min. The primary antibodies were used as follows: anti-AR: 1:100; anti-CD44, 1:200; anti-CgA, 1:1,000; anti-P53: 1:200; anti-PSA (Dako/A0562): 1:5,000; anti-NSE: 1:1,000; anti-CK5, 1:1,000; and anti-CK8, 1:1,000. The signal was detected with Dako Envision System Labelled Polymer HRP anti-mouse (cat 4001) or anti-rabbit (cat# 4003) for 30 min and developed with diaminobenzidine (DAB) for 10 min. Sections were counterstained with hematoxylin.

Western Blotting

Cultured cells were washed three time with cold phosphate-buffered saline (PBS) and lysed with lysis buffer (20 mM KCl, 150 mM NaCl, 1% NP-40, 1% Triton X-100, 50 mM NaF, 50 mM Tris, 1 mM DTT, 1 mM EGTA, 1× protease inhibitor, and 10% glycerol) for 15 min on the ice. The cells were homogenized and centrifuged for 30 min at 4°C. The protein concentration in the supernatant was determined with the Bio-Rad protein assay kit. Equal amounts of proteins were separated on 8% SDS–polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with non-fat dry milk for 1 hr, hybridized with primary antibody in non-fat dry milk overnight, washed with PBS for 30 min, incubated with secondary antibody for 1 hr, washed with PBS 1 hr, and detected with an ECL kit (Bio-Rad).

Quantitative RT-PCR

Total RNA was purified from cells with Fermentas Gene JET™ RNA purification Kit according to the manufacture's protocol. RNA was reverse transcribed by transcriptor reverse transcriptase (Fermentas RT-PCR Kit). The following specific forward and reverse primers were used: for NSE, 5′-GAGACAAACAG CGTTACTTAG-3′ and 5′-AGCTGCCCCTGCCTTAC-3′; for CgA, 5′-GCGGTGGAAGAGCCATCAT-3′ and 5′-TCTGTGGCTTCACCACTTTTCTC-3′; for CD44, 5′-AAGGTGGAGCAAACACAACC-3′ and 5′-TCCAC TTGGCTTTCTGTCCT-3′; for GAPDH, 5′-CATGGGT GTGAACCATGAGA-3′ and 5′-CAGTGATGGCATG-GACTGTG-3′.

Real-time PCR was performed with SA Biosciences RT^2 Real-time™ SYBR Kit. Total reaction volume was 15 μl and a cycle comprised of 95°C for 8 min, 95°C for 30 sec; for a total of 43 cycles followed by 95°C for 15 sec, 60°C for 1 min.

RESULTS

Expression of Luminal Differentiation Markers AR and PSA in LNCaP and PC3 Cells

The majority of clinically encountered adenocarcinomas of the prostate are acinar type adenocarcinomas which recapitulates normal acinar structures characterized by glandular formation. The tumor cells often have prominent nucleoli (Fig. 1A,B). In contrast, prostatic SCNCs show high-grade NE features including a diffuse, solid growth pattern, and a high mitotic rate. The tumor cells have a fine, homogeneous nuclear chromatin pattern, without prominent nucleoli or glandular formation (Fig. 1C,D).

An important feature of prostatic adenocarcinoma is that the tumor cells express markers characteristic of prostatic luminal cells such as AR and PSA. Many of the available PC cells lines, such as LNCaP, LAPC4,

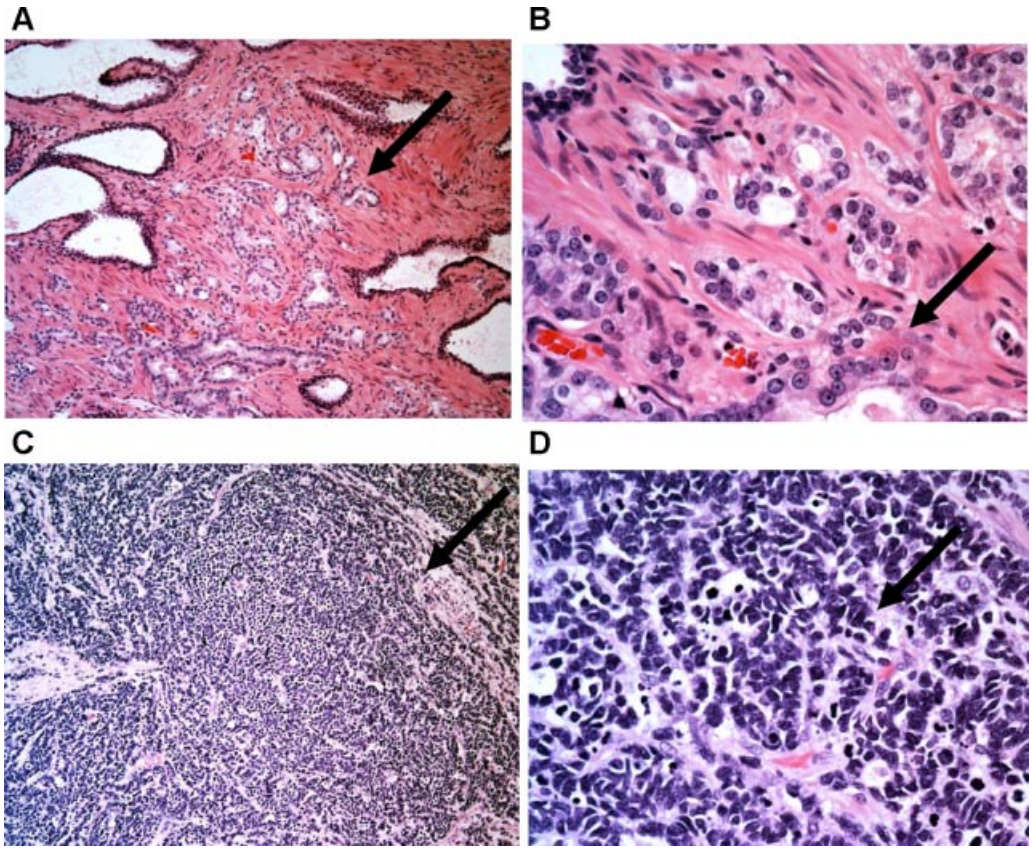

Fig. 1. Distinct histologic features of prostatic adenocarcinoma and SCNC. The upper panel (**A**,**B**) are low and high power pictures of prostatic adenocarcinoma with cancerous glands recapitulating the morphologic features of normal prostatic glands/ducts, and the tumor cells forming glandular structures. Note that many tumor cells show prominent nucleoli. The lower panel (**C**,**D**) are low and high power pictures of prostatic SCNC with high-grade neuroendocrine morphology including diffuse, solid growth pattern, high N/C ratio, fine nuclear chromatin pattern, and frequent mitotic figures. Note that there is no glandular formation and no prominent nucleoli (H&E 100× (A,C) and 400× (B,D)). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

22RV1, and VCaP, do show these features [21]. However, it is well known that PC3 cells are negative for such luminal differentiation markers [21], a feature that is shared by prostatic SCNC [9,10,13].

Immunohistochemical studies confirmed the previous reports and showed that while the cells of LNCaP xenograft tumors expressed AR similar to adenocarcinoma, the PC3 xenograft tumor cells did not express AR, similar to small cell carcinoma (Fig. 2A). Consistent with the above findings, Western blot assay showed that AR was expressed in LNCaP cells but not PC3 cells (Fig. 2B).

Decades of clinical observations have shown that prostatic adenocarcinoma responds to androgen deprivation therapy while the same therapy is not effective in prostatic SCNC [13], consistent with immunohistochemical finding showing that the former expresses AR while the latter is negative for AR. Growth assays showed that both types of cells proliferated well in media supplemented with normal FBS which contains steroid hormones including androgen. When cultured in media supplemented with charcoal-stripped FBS which is devoid of androgen, the proliferation of the AR positive LNCaP cells was significantly inhibited while the AR negative PC3 cells proliferated normally (Fig. 2C), supporting the notion that LNCaP cells are similar to adenocarcinoma cells and PC3 cells are similar to tumor cells of SCNC.

PSA expression is a hallmark of prostatic luminal cells and its expression is strictly controlled by AR activity. Immunohistochemical study shows that similar to cells of adenocarcinoma, LNCaP cells were positive for PSA while both SCNC and PC3 cells were negative for PSA (Fig. 3A), a finding that was confirmed by Western blot assay (Fig. 3B).

Expression of Neuroendocrine Markers in LNCaP and PC3 Cells

CgA and NSE are two of the markers commonly used to identify benign and neoplastic NE cells

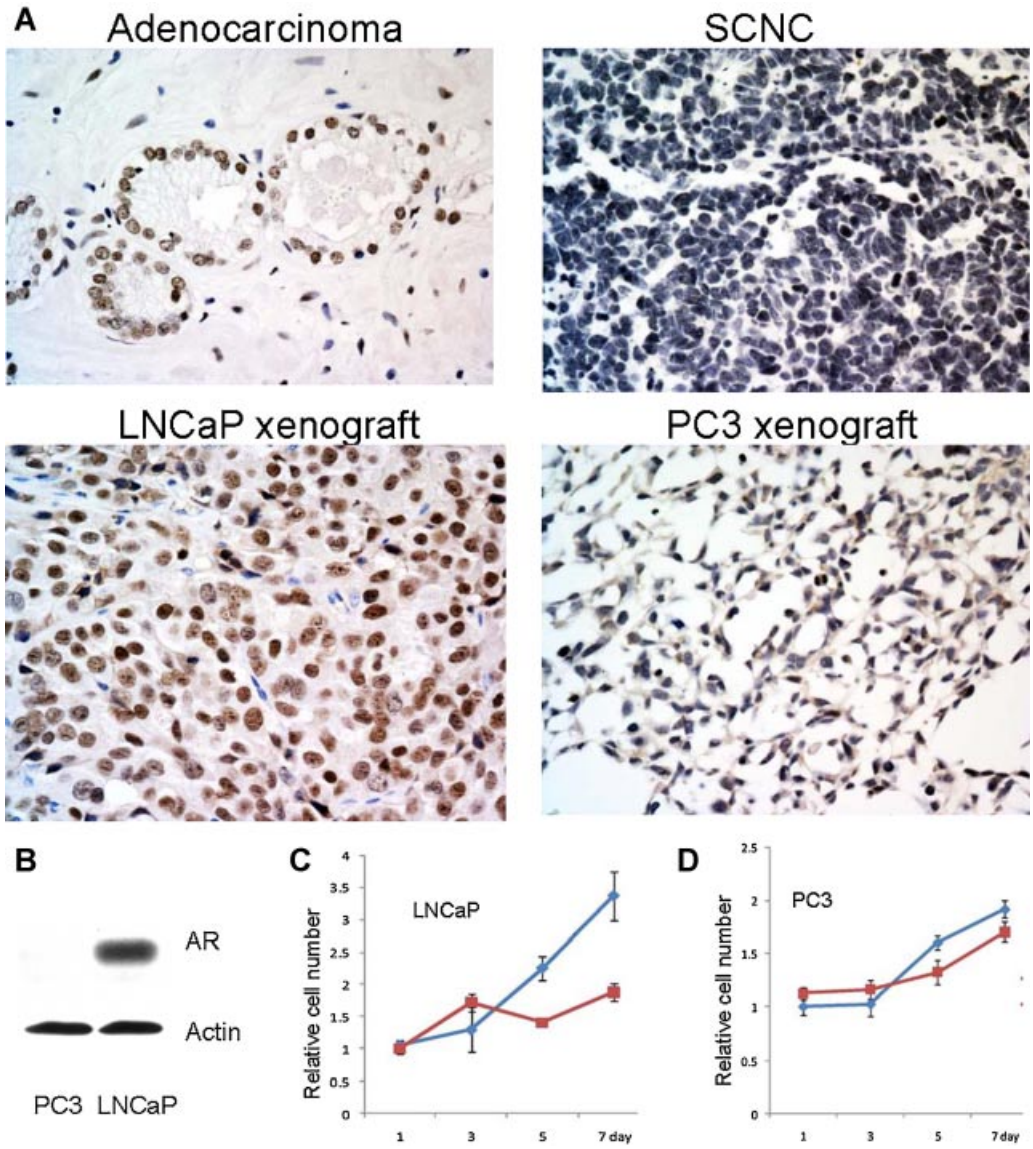

Fig. 2. Androgen receptor is expressed in prostatic adenocarcinoma and LNCaP cells but not in prostatic SCNC and PC3 cells. **A**: Immunohistochemical study with an anti-AR antibody shows positive staining in adenocarcinoma but not SCNC. The LNCaP xenograft tumor cells are positive for AR but the PC3 xenograft tumors are negative (IHC, 400× magnification). **B**: Western blotting shows that LNCaP cells express AR while PC3 cells do not. **C**,**D**: Growth assays show that withdrawal of androgen inhibits the growth of LNCaP cells but not PC3 cells. Blue line: media with FBS; red line: media with charcoal-stripped FBS. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

including those in the prostate [5]. RT-PCR and Western blotting studies showed that LNCaP cells, similar to the bulk, non-NE tumor cells of prostatic adenocarcinoma, were negative for the NE markers. In contrast, PC3 cells expressed high levels of these two NE markers (Fig. 4), consistent with previously published results [21,25–28]. Immunohistochemical studies showed that human prostatic adenocarcinoma cells were negative for NE markers NSE except the scattered NE cells, while SCNC showed extensive NE marker positivity (Fig. 5). Similarly, the LNCaP xenograft tumor cells were negative for NSE while PC3 xenograft tumor cells were positive (Fig. 5).

Expression of CD44 and Cytokeratins in LNCaP and PC3 Cells

CD44 is a putative cancer stem cell-associated marker in various human tumors [29]. Using a variety of cell lines and xenograft models of PC, the Tang's group showed that CD44 expression is associated with a small populations of tumor cells with

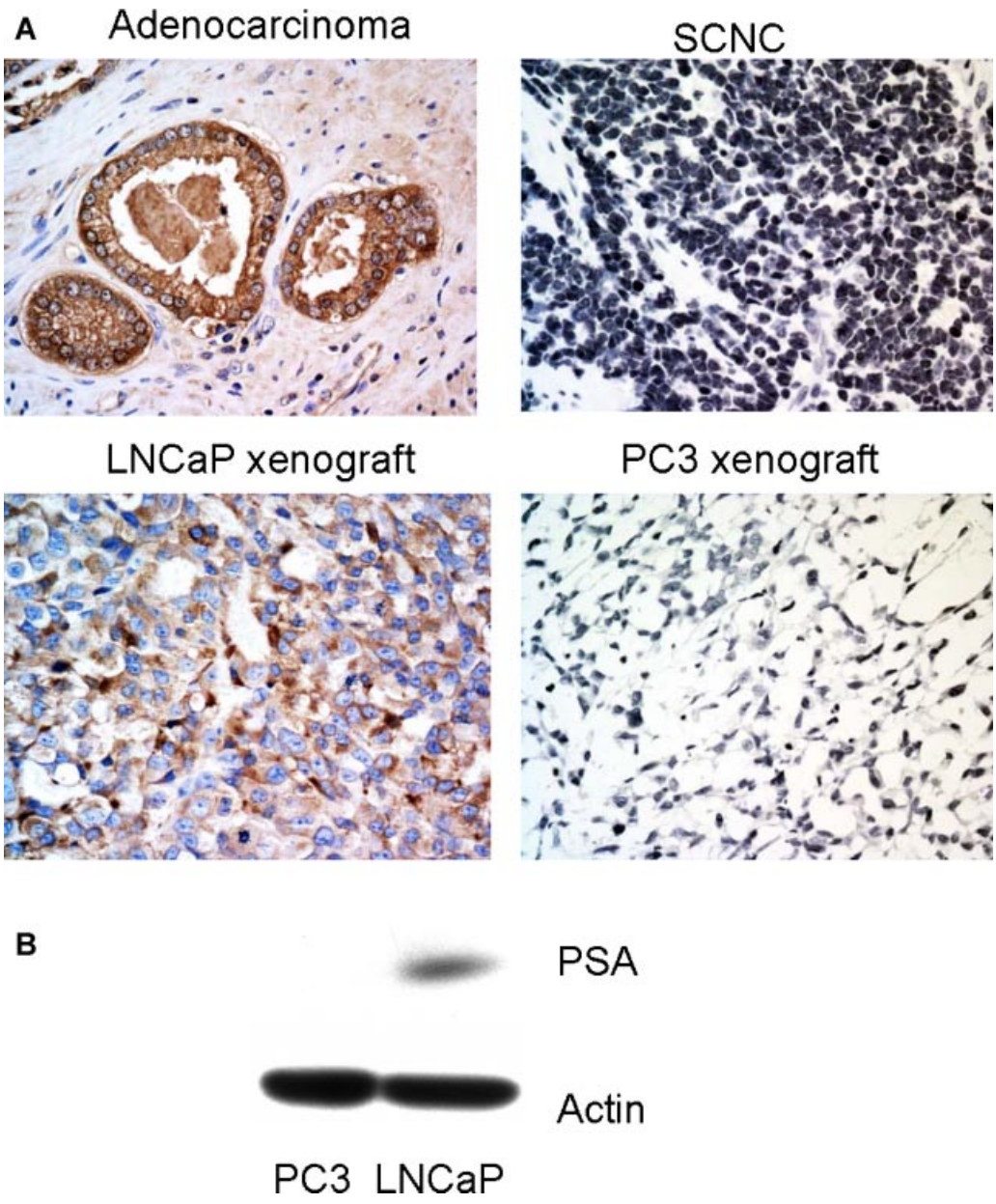

Fig. 3. PSA is expressed in prostatic adenocarcinoma and LNCaP cells but not in prostatic SCNC and PC3 cells. **A**: Immunohistochemical study with an anti-PSA antibody shows positive staining in prostatic adenocarcinoma but not SCNC. The LNCaP xenograft tumor cells are positive for PSA but the PC3 xenograft tumors are negative (IHC, 400×). B: Western blot analysis shows that LNCaP cells express PSA but the PC3 cells do not. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

increased tumorigenicity [30]. We have shown that in human prostatic adenocarcinoma, CD44 is selectively expressed in the scattered NE tumor cells but not in the bulk, luminal type tumor cells [28]. We have also shown that CD44 is commonly expressed in NE tumor cells of prostatic SCNC [24]. Immunohistochemical studies showed that LNCaP xenograft tumor cells were negative for CD44, similar to the bulk tumor cells of prostatic adenocarcinoma, while PC3 xenograft tumor cells expressed CD44 strongly and diffusely, similar to tumor cells of SCNC (Fig. 6A). Quantitative RT-PCR study in cultured cells confirmed the above findings (Fig. 6B).

Benign prostate glands contain basal and luminal cells and the two types of cells have distinct cytokeratin profiles. CK5 and CK14 are considered basal cytokeratins while CK8 and CK18 are luminal cytokeratins [31,32]. Prostatic adenocarcinomas express luminal cytokeratins but not basal cytokeratins [31,32]. It has been reported that while the tumor cells of prostatic adenocarcinoma show a diffuse and strong staining pattern for CK8, those of prostatic

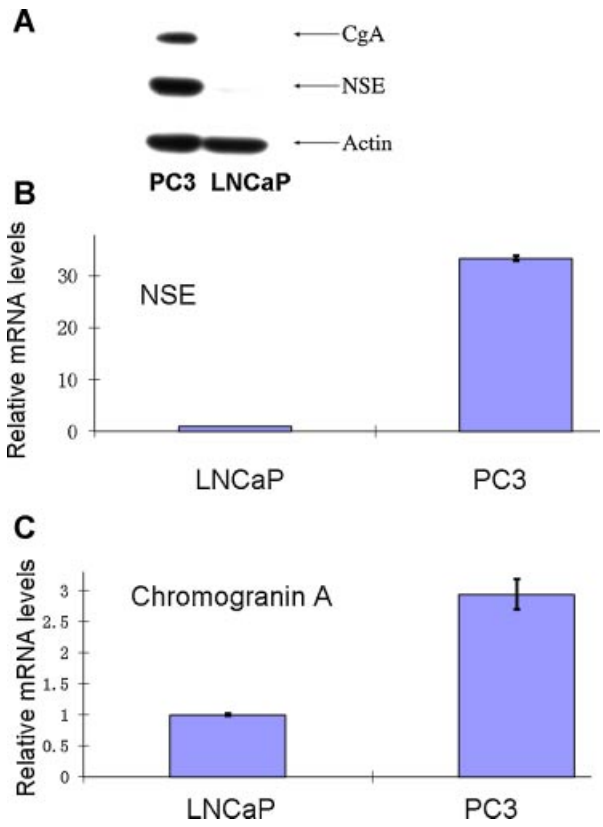

Fig. 4. PC3 cells express NE cell markers but LNCaP cells do not. **A**: Western blot assay shows that PC3 cells, not LNCaP cells, express NE markers chromogranin A and NSE. **B**,**C**: show the results of qPCR studies showing higher mRNA levels for CgA and NSE in PC3 cells than in LNCaP cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

SCNC show focal and dot-like staining pattern [33]. We compared the cytokeratin profiles of LNCaP and PC3 cells with those of adenocarcinoma and SCNC and showed that both cells were negative for basal cell cytokeratin CK5 as expected (data not shown). IHC staining for luminal cytokeratin CK8 showed strong and diffuse staining in LNCaP cells similar to adenocarcinoma cells, while PC3 cells showed focal positive staining similar to SCNC cells, further demonstrating that PC3 cells have features characteristic of prostatic SCNC (Fig. 7).

DISCUSSION

Various experimental models have proven extremely useful in studying many important aspects of PC such as etiology, molecular pathways of signal transduction, imaging, prevention, and therapy. Cell lines were the dominant experimental models for many years and are still the main tools used in many of today's publications even after animal models have become widely available in recent years. Cell lines provide many unique advantages, such as being easy to grow with commonly available reagents and equipment, the ability to grow large amount of materials for many experiments in a short period of time, and the resulting material being pure populations of cells suitable for molecular biology and biochemical studies. LNCaP and PC3 have been the most commonly used PC cell lines but they possess significantly different characteristics, the most important being that LNCaP cells express luminal differentiation markers AR and PSA while PC3 cells do not. Consequently, LNCaP cells are androgen-dependent, and androgen withdrawal inhibits their growth, while PC3 cells are androgen-independent and proliferate normally in androgen-deprived media. In addition, xenograft tumors of LNCaP are slow growing and less invasive, while PC3 xenograft tumors proliferate rapidly and are more invasive. In in vitro assays, PC3 cells possess higher migratory capability than LNCaP cells. As a result, some researchers consider LNCaP and PC3 cells to represent less aggressive and more aggressive forms of prostatic adenocarcinoma, respectively. In some publications, the two cell lines have also been used to represent androgen-dependent and castration-resistant PCs, respectively. Therefore, certain molecular differences between the two cell lines have been considered to be responsible for the aggressiveness or the progression of prostatic adenocarcinoma.

The above notion is inconsistent with histologic observations in human tumors. Gleason grading has been one of the most useful histologic parameter in predicting the biologic behavior and clinical outcomes of prostatic adenocarcinoma. Prostatic adenocarcinoma, which comprises over 90% of all PCs, nearly always expresses AR and PSA irrespective of Gleason grade, so do the vast majority of castration-resistant PCs [34–38]. The recently reported efficacy of MDV3100 and Abiraterone in treating castration-resistant PC further supports the notion that in the late stage of PC, AR is still expressed, being functional and critical for the survival of the tumor cells [39]. Therefore, having a cell line that is negative for AR and PSA to represent an aggressive type or a castration-resistant form of prostate adenocarcinoma is problematic and likely misleading.

On the other hand, rare cases of PCs are variant forms including prostatic SCNC which shows NE phenotype. Prostatic SCNC is extremely aggressive and usually causes deaths in months. It is different from prostatic adenocarcinoma in that the tumor cells do not express luminal differentiation markers AR, PSA and are not responsive to hormonal therapy which provides short-term benefits in nearly all patients with advanced and metastatic prostatic

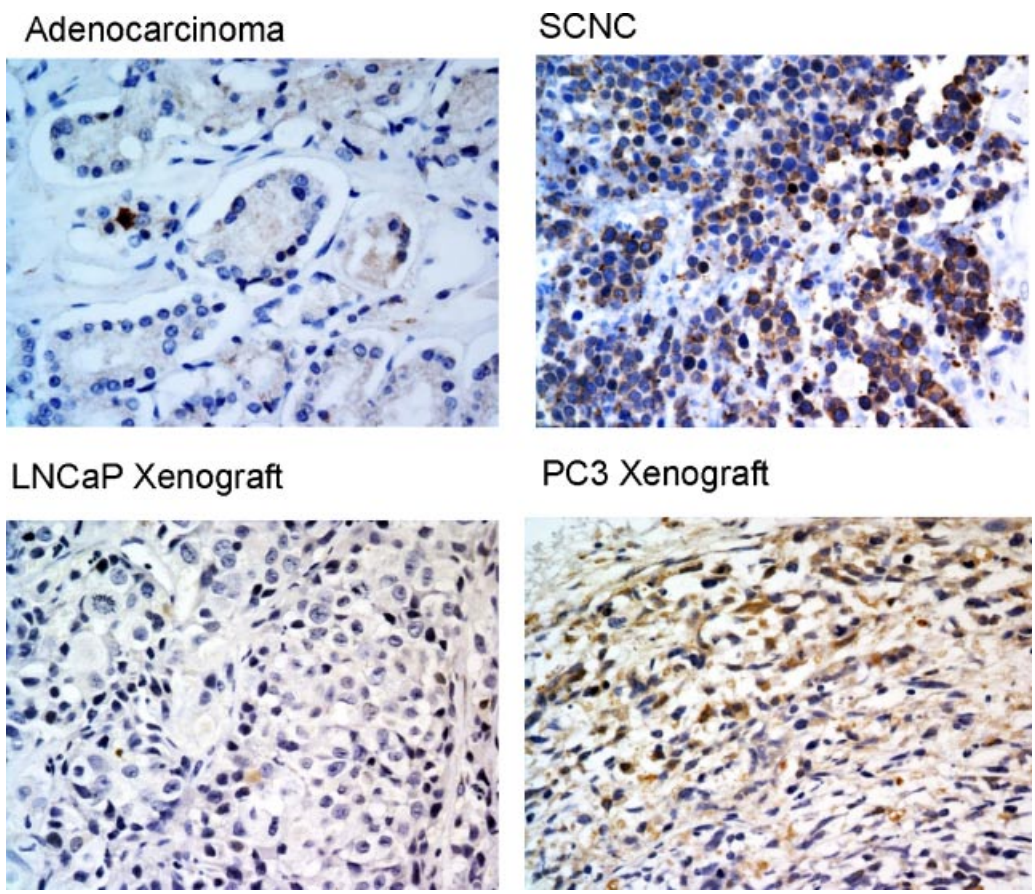

Fig. 5. Neuroendocrine marker NSE is expressed in prostatic SCNC and PC3 cells but not in prostatic adenocarcinoma and LNCaP cells. Immunohistochemical study with an anti-NSE antibody shows positive staining in prostatic SCNC but not in adenocarcinoma. The LNCaP xenograft tumor cells are negative for NSE but the PC3 xenograft tumors are positive (IHC, 400×). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

adenocarcinoma. Therefore, SCNC is an entirely different disease than prostatic adenocarcinoma and it is important to distinguish the two entities clinically and pathologically for treatment and prognosis purposes [13].

Here we have provided convincing evidence demonstrating that LNCaP cells have features of prostatic adenocarcinoma including the expression of luminal differentiation markers AR and PSA, while PC3 cells have features typical of prostatic SCNC such as being negative for AR and PSA but positive for NE markers. Consistent with these observations, androgen withdrawal induces growth inhibition of LNCaP cells but not PC3 cells. It must be noted that androgen withdrawal can induce NE differentiation in LNCaP cells [40], and may also do so in human adenocarcinoma [41,42], while the NE phenotype and other features similar to SCNC observed in PC3 cells are present under normal culture conditions without any external stimuli [21,25–28].

The patterns of expression of CD44, a putative cell surface marker for normal and cancer stem cells including PC stem cells, are also consistent with our conclusion. Tang's group has shown that expression of CD44 identifies PC cells with increased tumor initiation potential [30]. Both his group and ours have observed that while LNCaP cells do not express CD44, nearly 100% of PC3 cells express this marker [28,30]. We have also demonstrated that CD44 is not expressed in human prostatic adenocarcinoma (with the exception of the rare NE tumor cells) [28] but it is strongly and diffusely expressed in human prostatic SCNC [24], further supporting our conclusion.

The cell of origin for prostatic adenocarcinoma remains controversial. Traditionally luminal cells are considered to be the likely origin because the precursor of prostatic adenocarcinoma (high-grade prostatic intraepithelial neoplasia, or HGPIN) is characterized by cytologic atypia in luminal cells histologically. This theory is supported by a recent study in an animal model [43]. However, other studies have shown that basal cells are the likely cells of origin for adenocarcinoma [44–46]. Nevertheless, the bulk tumor cells of adenocarcinoma share features of luminal cells such

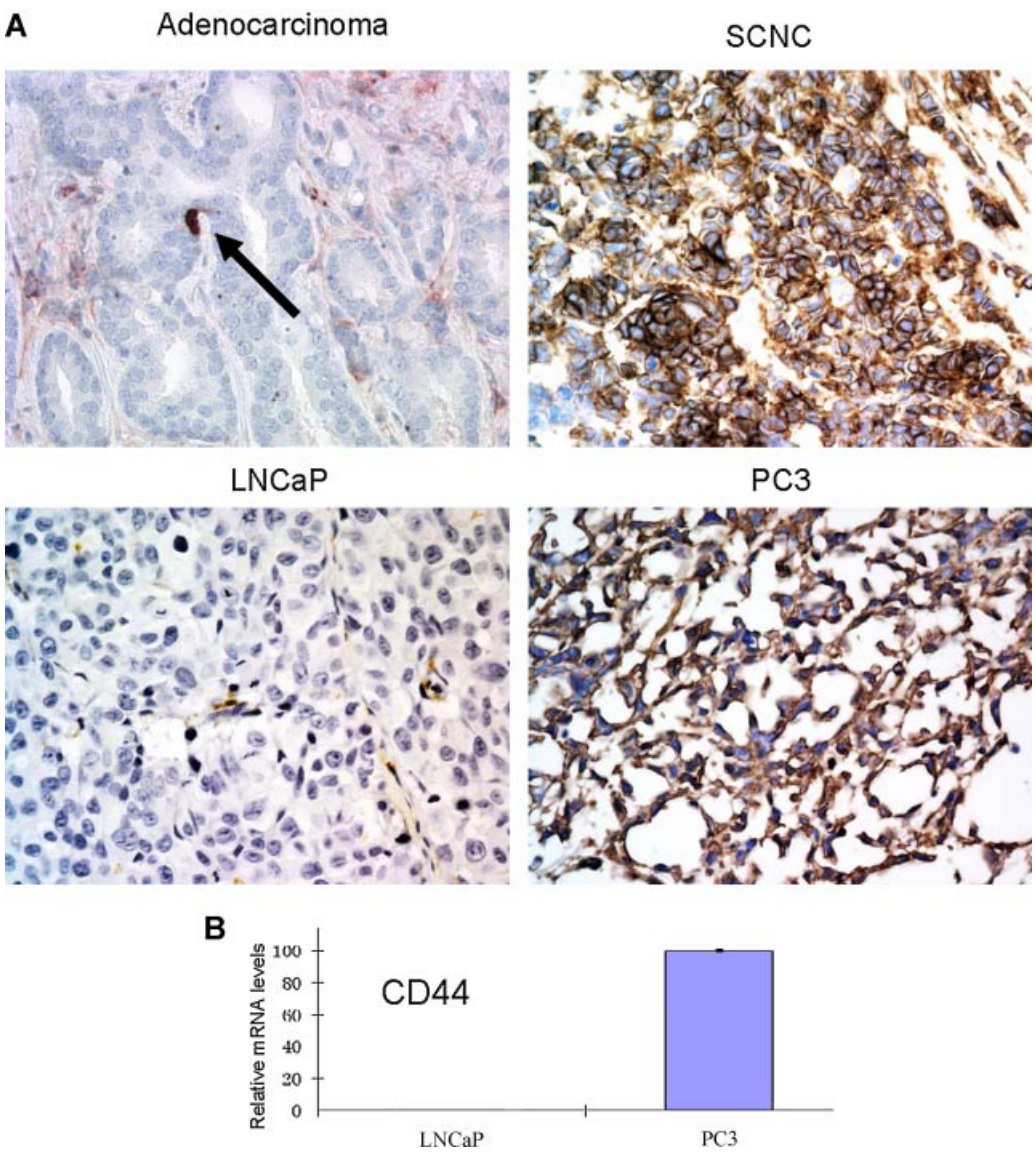

Fig. 6. CD44 is expressed in prostatic SCNC and PC3 cells but not in the luminal type tumor cells of prostatic adenocarcinoma and LNCaP cells. **A**: Immunohistochemical study shows positive staining in prostatic SCNC but not in adenocarcinoma (with the exception of rare NE tumor cells, arrow). The LNCaP xenograft tumor cells are negative for CD44 but the PC3 xenograft tumors are positive (IHC, 400×). **B**: qPCR study shows very low levels of CD44 mRNA in LNCaP cells but high levels in PC3 cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

as the expression of AR, PSA, and luminal cytokeratins, as do LNCaP cells. The cell of origin for prostatic SCNC is even less clear. The tumor cells of prostatic SCNC do not express basal cell markers or luminal cell markers AR and PSA. In addition, anti-CK8 staining shows focal, dot-like staining pattern, a featured characteristic of small cell carcinoma [13]. PC3 cells share all the characteristics of prostatic SCNC.

Results similar to what we have presented in this manuscript have been reported from time to time in various publications and represent well-established facts widely known to PC researchers. In this manuscript, we have for the first time put all of them together in a rational manner and arrived at the important conclusion that PC3 is likely a cell line of prostatic SCNC. At the time of manuscript preparation, a Pubmed search using ''prostate AND PC3 or PC-3'' returned more than 5,000 articles all related to PC. It can be assumed that in all of those studies, PC3 cells were considered to represent an aggressive form or advanced (castration-independent) stage of prostate adenocarcinoma. Our observations and conclusions have significant implications as molecular mechanisms and therapeutic efficacies observed with PC3 cells are likely applicable to prostatic SCNC but not to the adenocarcinoma.

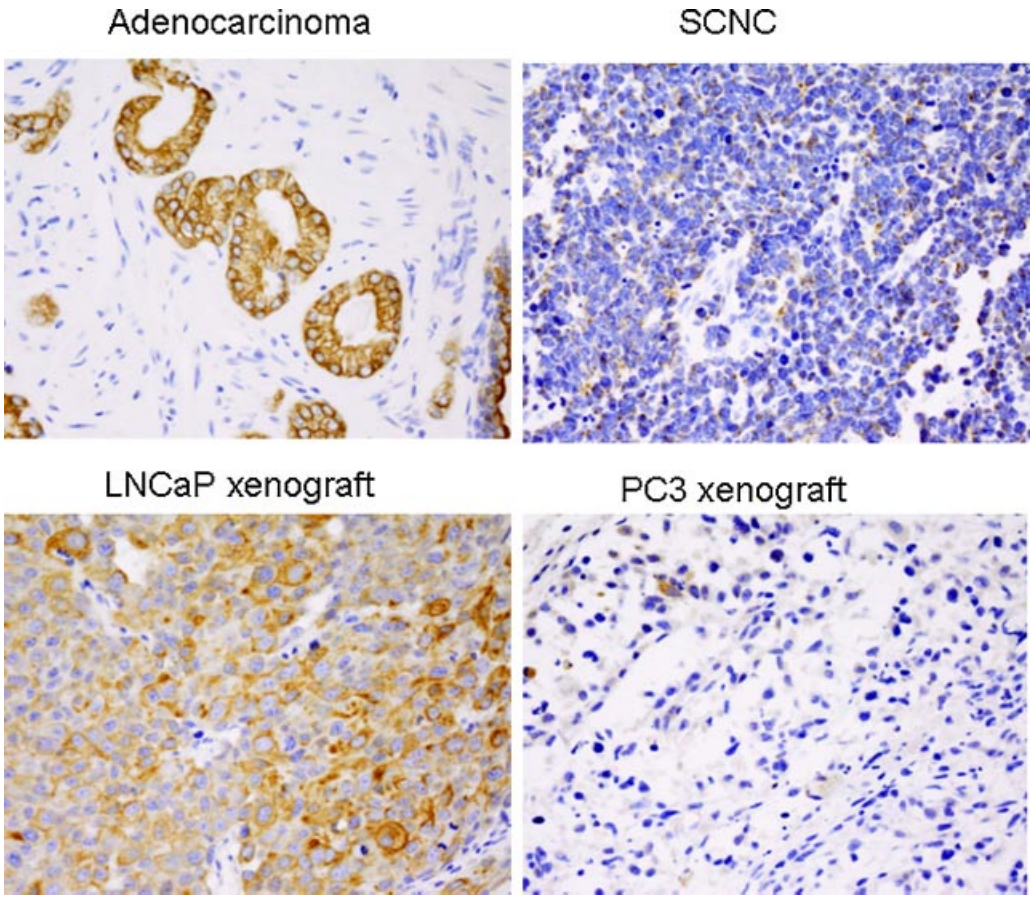

Fig. 7. CK8 is strongly and diffusely expressed in prostatic adenocarcinoma and LNCaP cells but only focally and weakly in SCNC and PC3 cells. Immunohistochemical study with an anti-CK8 antibody shows positive staining in prostatic adenocarcinoma cells but a dot-like staining pattern in tumor cells of SCNC. The LNCaP xenograft tumor cells are diffusely positive for CK18 but the PC3 xenograft diffuse tumors are only focally positive (IHC, 400×). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Despite all the recent progresses, LNCaP and PC3 cells, with features characteristic of adenocarcinoma (AR+, PSA+, androgen-dependent, diffuse positivity for CK8) and SCNC (NE markers+, castration-resistant, CD44+, focal positivity for CK8), respectively, will continue to help us understand the molecular mechanisms of the two different tumors and aid in our effort in developing novel therapies. The main objective of our manuscript is to point out to investigators that PC3 cells should not be considered a cell line representing prostatic adenocarcinoma, a notion that has been held ever since this cell line was established more than 30 years ago.

CONCLUSION

We conclude that while LNCaP cells have features of prostatic adenocarcinoma, PC3, a cell line often considered to represent a more aggressive form or castration-resistant PC, actually shares important features with prostatic SCNC, a conclusion that has important implications to the field of PC research.

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